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14. ABSTRACT
This final report for the Physician Research Training Award summarizes work on the genetic analysis of circulating hormone refractory prostate cancer micrometastases. As metastatic tissue is often inaccessible in advanced prostate cancer patients, analysis of circulating tumor cells may provide understanding of the biology of hormone refractory prostate cancer as well as chemotherapy resistance. Oligonucleotide array comparative genomic hybridization allows the assessment of genetic changes that may occur in the process of metastasis and chemotherapy resistance. Genomic profiling using this technology will go beyond cell counting, and circumvent technical complexities related to working with RNA. Using the Vitatex cell isolation system, preliminary data suggested that reproducible genomic alterations are observed in the circulating tumor cells isolated from patients with metastatic hormone refractory prostate cancer.

15. SUBJECT TERMS
circulating tumor cells, prostate cancer, chemotherapy resistance, genomic analysis

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Introduction

This project focuses on the genetic analysis of circulating hormone refractory prostate cancer micrometastases with the goal of identifying mechanisms of chemotherapy resistance. Hormone refractory prostate cancer (HRPC) metastatic tissue is difficult to obtain for research as most metastatic sites are not conducive to biopsy. However, circulating tumor cells (CTC's) have been found in high numbers in patients with metastatic HRPC. CTC's represent an untapped resource for studying the genetics of metastatic HRPC. These cells are easily accessible in the peripheral blood. The purpose of this research is to detect genetic alterations that occur during the development of chemotherapy resistance, to give insight into the mechanisms behind this resistance, and determine potential therapeutic strategies to combat it. To accomplish this, we have refined the techniques needed to isolate CTCs and genomic DNA from those cells, amplify the DNA if necessary, and evaluate genomic alterations using oligonucleotide comparative genomic hybridization (oCGH). Frequently used CTC isolation technologies (e.g. Veridex) do not allow for highly efficient interrogation of DNA because the viable CTCs are not recovered in sufficient purity. We sought to use a technique that would allow us to go beyond CTC enumeration. Our results using the Vitatex technology to capture living CTCs suggest that this approach is feasible and cost efficient. This technology will be incorporated into an upcoming phase II study of second-line chemotherapy for hormone refractory prostate cancer to investigate the "lethal phenotype" of prostate cancer. We hypothesized that the copy number changes could be prognostic and aid in future chemotherapy regimen selection.

After moving to a new institution, several new projects were considered to evaluate biomarkers of chemotherapy resistance and association of genetic changes with outcome in circulating tumor cells. These projects met with significant technical and biological obstacles. This report will summarize the grant findings and obstacles.

Tasks 1, 2: *Isolation and characterization of circulating micrometastases of chemotherapy naïve and chemotherapy resistant HRPC.*

Procedures and techniques to capture circulating cells using cell-adhesion matrices (CAM) have been continually optimized. This has taken a significant amount of effort and time. The methodology of DNA amplification for CTC DNA was piloted and preliminary data demonstrates the methodology has good fidelity compared with unamplified DNA. In addition, further experiments evaluating genomic changes in CTCs from HRPC patients show promising results, described below.

Most prior studies involving CTCs in prostate cancer patients have been enumeration studies or gene expression studies. Gene expression is dependent on RNA extraction procedures and on environment. Therefore, the disparate published results may be related to minor differences in RNA isolation techniques and the environment of the cells prior to and after isolation. Expression profiles of CTCs may share only limited concordance with cells from the primary tumor and significant variation within and between patients is expected. Genomic profiling will go beyond cell counting, and circumvent technical complexities related to working with RNA.

Currently oligonucleotide comparative genomic hybridization (oCGH) requires 500ng of input DNA. However, the amount of DNA isolated from circulating tumor cells may be less than 500ng. Because the same issue confronts clinical application of array CGH, the Paris/Collins laboratory has been evaluating linear and rolling circle methods for the isolation of DNA from formalin-fixed paraffin-embedded (FFPE) biopsy specimens.[1] Data suggests that it is possible to obtain DNA from paraffin that works very well for array and oligonucleotide CGH and that whole genome amplification (WGA) does not introduce unacceptable copy number artifacts as determined using array CGH. Similar oCGH profiles obtained with unamplified and matching WGA amplified FFPE prostate DNA. Therefore, if necessary, extraction of DNA from circulating tumor cells followed by whole genome amplification should provide sufficient high quality DNA for use with oCGH. FFPE biopsy samples can be treated similarly, if needed. These methodologies were refined over the last year and are now able to be applied to DNA isolated from CTCs.

We have been able to extract on average 7 micrograms of DNA (range 1 μg -16 μg) from isolated cells taken from 20 mL of peripheral blood for use in genomic analysis. A total of 14 patients have been collected to date, and we have been able to isolate CTC DNA from 9 of those patients. Now that technical details have been worked out and preliminary results have been obtained (see below) suggesting that we are able to isolate CTCs using this technology, we are planning on prospectively collecting CTCs in patients enrolling on the phase II component of NCI7347, “A phase I/II study of ixabepilone, mitoxantrone, and prednisone in patients with metastatic hormone refractory prostate cancer previously treated with chemotherapy.” The study will enroll 58 patients, and we expect that, based on previous experience with this isolation technique and prior data regarding the prevalence of CTC’s in patients with metastatic HRPC patients, approximately 40 patients will have suitable CTC genomic DNA for analysis.

Tasks 3,4,5

Analyze and compare gene signatures of circulating tumor cells to biomarkers previously identified, identify markers of chemotherapy resistance and response in CTC's in HRPC.

During the time period in which we demonstrated that CTC DNA can be isolated from whole blood of prostate cancer patients, the Paris/Collins laboratory switched to the Agilent oCGH platform because it offers comparable data to the BAC arrays, but at a much higher resolution (9kb versus 1.4Mb) and works well with smaller amounts of DNA (500ng).[1] As a result, the Agilent oCGH platform was utilized for the CGH studies.

Data has been generated from 9 samples for which DNA was able to be isolated from CTCs. White blood cells (WBCs) were collected from each patient. Three matched CTC and WBC samples were profiled and in each case the percentage of the genome that was altered in the CTC DNA was larger. These data are presented in Table 1. The frequency of DNA copy number changes observed in the CTC's is shown in Figure 1. Recurrent alterations are being identified in different CTCs from different patients, suggesting that genes may be present at the identified loci that are involved in HPRC pathogenesis. Prospectively collected specimens from a uniformly treated patient population as part of the phase II study described in Task 1 will be analyzed over the next year to elicit statistically meaningful prognostic DNA based biomarkers.

Two of the patients (#8 and #13) had tissue available from their radical prostatectomy (RP) procedure. High volume tumor areas were macrodissected with the assistance of a pathologist, Dr. Jeffrey Simko, and DNA extracted. The RP DNA was profiled on the Agilent arrays and compared to the matched CTC copy number profile. The Kappa score, an indication of the correlation between each of the two profiles, was comparable for each set (Table 2).

To further support the identity of the isolated cells as CTCs, we have collaborated with Dr. Wen-Tien Chen at SUNY Stonybrook who has conducted extensive experiments spiking PC3 (prostate cancer cell line) cells into whole blood of a healthy donor, and demonstrated high efficiency recovery using the CAM Vitatex system. In addition, his laboratory has enumerated CTCs in blood samples from 27 patients with metastatic prostate cancer. The number of CTC recovered in the blood averages over 200 CTC/mL. A manuscript has been published and is attached.

Previous work enumerating CTCs in the blood of patients with prostate cancer done in collaboration with Dr. John Park and Dr. Jorge Garcia has been published (see attached manuscript).

In related work, Dr. Rosenberg collaborated with Dr. Paris to utilize a library of 44 specimens obtained from patients with HRPC – a unique resource with the potential to be leveraged for the identification of novel genomic pathways associated with castration resistance. Novel pathway identification is a high priority in HRPC. Multiple novel therapeutic agents and strategies are in development creating the major challenge of matching an investigational agent to the biological pathways that are active in a given

clinical state of disease. We hypothesized that genome copy number profiles can be used to define the mechanisms of disease in HRPC. Array comparative genomic hybridization (aCGH) is a powerful tool for biomarker discovery and identification of genes involved in cancer progression because it allows high resolution and quantitative detection of copy number aberrations in tumor genome that can be associated with clinical outcome.[2-4] Recurrent deletions and amplifications reveal loci encoding tumor suppressor genes and oncogenes, respectively, and their identification is expedited by using the human genome sequence. More recently, oligonucleotide CGH has allowed for higher resolution copy number profiles.

Task 6. Ongoing months 42-48

Task 6A. To determine whether the presence of TMPRSS2:ERG fusion in castration resistant prostate cancer circulating tumor cells is associated with a shorter overall survival prognosis.

Background/rationale

The finding of the TMPRSS2:ERG fusion in prostate cancer identified a distinct molecular subtype. TMPRSS2 fusions appear to be maintained through the clonal evolution of prostate cancer through castration resistant prostate cancer (CRPC). The biological effects of these fusion proteins are still the subject of investigation, with little being understood about their precise role in prostate cancer. Similarly, the impact of TMPRSS2 fusion on the prognosis of patients with CRPC is undefined. Obtaining CRPC tumor tissue in large numbers of CRPC patients is difficult due to the inaccessibility of tissue for biopsy. Therefore, circulating tumor cells (CTCs), which are easily accessible in the peripheral blood of CRPC patients, may provide an acceptable surrogate to interrogate for the presence or absence of the fusion transcript. Previous work at the DFCI has established that the detection of the fusion transcript is feasible in the peripheral blood of patients with prostate cancer (unpublished data).

After review of the clinical data and the timing of the blood samples, only small fraction of the 362 patients initially identified as having CRPC within the database had blood specimens obtained during the time following resistance to androgen deprivation therapy. The sample size was insufficient to continue this experiment.

Task 6B. To determine whether the presence of CpG island hypermethylation in the multidrug resistance gene (MDR1) promoter is associated with docetaxel chemotherapy sensitivity in CRPC patients treated with docetaxel.

Background/Rationale

The multidrug resistance (*MDR1*) gene product is a membrane protein that functions as an ATP-dependent exporter of drugs from cells. MDR1 (or ABCB1, p-glycoprotein) has been implicated in docetaxel resistance. Polymorphisms in MDR1 are implicated in docetaxel sensitivity, and efficacy of taxanes are reduced in tumors that overexpress MDR1.[5, 6-8] Similarly, mechanisms that reduce MDR1 expression are hypothesized to increase sensitivity to docetaxel chemotherapy.

CpG island hypermethylation at various genetic loci are frequent in prostate cancer. Promoter hypermethylation inhibits transcriptional activity of the downstream gene, leading to reduction of mRNA expression and protein production. Hypermethylation of the promoter of the MDR1 gene is associated with prostate cancer, and appears to be more frequent in patients with metastases than with recurrent or localized prostate cancer.[9, 10] Cell-free circulating DNA is detectable in prostate cancer, and alterations observed in free-DNA reflect alterations present in the primary and metastatic tumors.[11] The origins of this DNA are not completely understood, but apoptotic and necrotic cell death of cancer cells appear to contribute. Preliminary data suggested that CpG hypermethylation could be detected in the promoter of the MDR1 gene in the serum of patients with CRPC. However, the volume of serum required to do this is considerable (>500 uL), and after pilot studies, this approach was abandoned due to the need to conserve this precious resource.

A similar project was conceived that would evaluate the expression of alternative splice variants of the androgen receptor to determine the impact of outcomes in CPRC. Similar to the TMPRSS2:ERG fusion, a sufficient number of appropriate samples were unable to be identified. In addition, preliminary work suggested that these transcripts were a minor species of AR and not able to be detected by RT-PCR in human specimens.

Task 7

Educational component

While at UCSF, Dr. Rosenberg met regularly with Dr. Small to discuss research and clinical trial design, as well as with Dr. Paris to discuss progress on CTC isolation and characterization. At the Dana-Farber Cancer Institute, Dr. Rosenberg participates in weekly protocol development meetings, the Kantoff lab meetings, and biweekly seminar series. He is involved in teaching and mentoring fellows. In addition, Dr. Rosenberg has been named to the CALGB Genitourinary Oncology Core Committee, and is involved in the decision-making for the new and ongoing clinical trials and translational research of the Committee. At the CALGB, Dr. Rosenberg leads the bladder cancer cadre.

Table 1: Comparing the number of copy number changes in matched CTC and WBC samples

Samples	Percentage of the genome that is aberrant
CTC7	3.21%
WBC7	0.97%
CTC8	2.04%
WBC8	0.60%
CTC13	1.26%
WBC13	0.85%

Table 2. The correlation score for the copy number profiles of matched CTC and radical prostatectomy (RP) samples.

Matched Pairs	Kappa Value
CTC8 and RP8	0.9873
CTC13 and RP13	0.8346

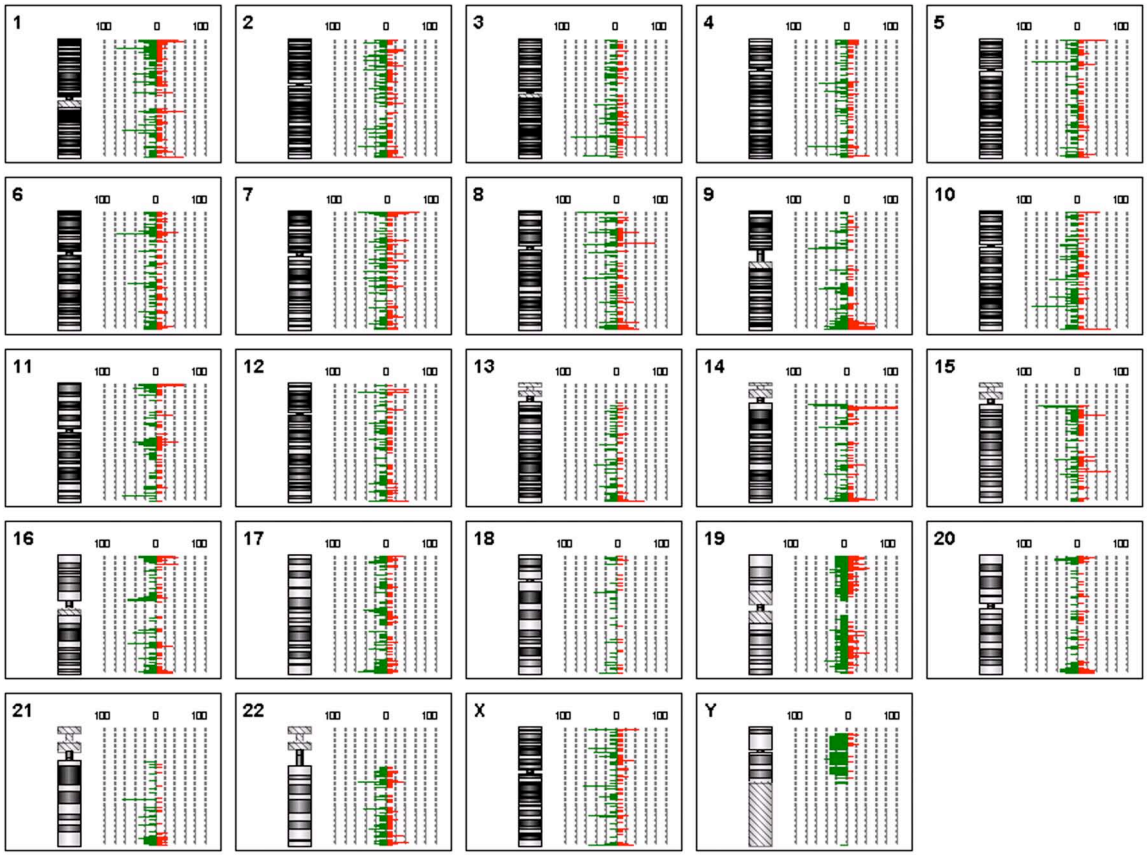

Figure 1. Frequency plot for the nine CTCs is plotted against the chromosomal position. The frequency of gains is shown in red for each chromosome and the frequency of deletions is shown in green, each ranging from 0-100% in 20% intervals. Note the recurrent changes between patients may represent loci associated with HRPC.

Key Research Accomplishments:

- Demonstration that reproducible genomic changes can be observed in CTCs using the Vitatex isolation technology.
- Obtained funding for HRPC tissue-based study to investigate oCGH changes and identify new pathways

Reportable Outcomes:

1. Harzstark AL*, Rosenberg JE*, Weinberg VK, et al. Ixabepilone, mitoxantrone, and prednisone for metastatic castration-resistant prostate cancer after docetaxel-based therapy: a phase 2 study of the department of defense prostate cancer clinical trials consortium. Cancer 2011: Epub ahead of print. *contributed equally
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Conclusions:

We have demonstrated that the Vitatex technology can be used to isolate CTCs for genomic analysis. Confirmatory experiments have been conducted by collaborators. High quality DNA is able to be isolated from these cells. oCGH using CTC DNA isolated by the Vitatex system suggests that recurrent genomic alterations are present in CTCs. Specimen collection will continue as part of a prospective clinical trial of HPRC patients. Once sufficient numbers of specimens have been obtained, we will be able to begin to evaluate the genomic alterations associated with CTCs in HRPC in general, and chemotherapy resistance in particular. The Agilent array technology is high resolution allowing the identification of specific genes that may be altered in metastatic and chemotherapy refractory HRPC.

Other projects have been less successful at achieving reportable results. Moving institutions, technical concerns, and lack of adequate specimens have limited the ability to produce results for the final six months of the grant.

<u>Personnel Supported</u>

Jonathan Rosenberg, MD
Pamela Paris, PhD

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Appendix

Urological Oncology

The urological oncology section is relatively long this month, and this reflects the many high-quality manuscripts we receive. When you consider our relatively high rejection rate, you will understand just how many papers on this topic are submitted. The high quality of oncology papers is clear in this month's section. You will also notice that all but one of them are on prostate cancer, and the reason for this is similar to that mentioned above, as this topic is, as might be expected, the most commonly submitted in this section. However, I am only too happy to reassure readers, and those primarily interested in other types of urological cancer, that the imbalance in this month's section is not a permanent fixture.

Evaluation and significance of circulating epithelial cells in patients with hormone-refractory prostate cancer

Jorge A. Garcia*, Jonathan E. Rosenberg*, Vivian Weinberg†, Janet Scott*, Mark Frohlich*, John W. Park* and Eric J. Small*†‡

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Accepted for publication 21 September 2006

OBJECTIVE

To determine the feasibility of using flow cytometry fluorescence-activated cell sorting (FACS) analysis for detecting circulating epithelial cells (CECs) in patients with hormone-refractory prostate cancer (HRPC), and to determine whether CECs can be used to predict survival in these patients.

PATIENTS AND METHODS

Several prognostic models that include routinely used clinical and laboratory variables for predicting survival in men with HRPC have been reported; the presence of CECs measured by reverse transcriptase-polymerase chain reaction for prostate-specific antigen (PSA) in patients with HRPC is an independent prognostic factor for survival. CECs detected by FACS analysis correlate with advanced stage and poor survival outcome. A retrospective study was conducted to assess the presence of CECs by FACS analysis in metastatic HRPC patients initiating systemic chemotherapy with a taxane-based regimen. The association between clinical variables previously described and the presence of CECs along with the effect of the magnitude of CECs on survival was calculated, in 41 patients with HRPC, all of whom had peripheral blood collected for FACS analysis.

RESULTS

Except for four patients, all those with metastatic HRPC had detectable CECs. Among these patients, the number of CECs/mL was correlated with age, serum PSA level and serum alkaline phosphatase (ALP). Higher serum levels of PSA and ALP predicted a poor survival outcome. Similarly, patients with ≤1.8 CECs/mL had a significantly longer survival than those with more CECs/mL ($P = 0.02$). With a median follow-up of 15.4 months, the median overall survival for all patients was 18.4 months.

CONCLUSIONS

The presence of more CECs in patients with metastatic HRPC was associated with a poorer survival outcome; levels of ≥1.8 CECs/mL were associated with a shorter survival in patients with metastatic HRPC.

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KEYWORDS

hormone-refractory prostate cancer, circulating epithelial cells, flow cytometry, fluorescence-activated cell sorting, chemotherapy

INTRODUCTION

Prostate cancer remains the most common cancer among men in the USA, accounting for >32% of all male malignancies. It is estimated that >234 000 men will be diagnosed with prostate cancer during 2006, and 27 350 will die from the disease. Virtually all deaths are due to the development of hormone-refractory prostate cancer (HRPC) [1]. Several prognostic models predictive of survival in men with HRPC have been reported [2–5]. Numerous reports have suggested that early-stage cancers have the potential to begin shedding cancer cells into the circulation early in their development. Unfortunately, the natural history of these cells, their ability to establish metastases, and their role in disease recurrence remains unclear. Detection of micrometastases, or circulating tumour or epithelial cells (CECs) has become an attractive technique that can be used to assess the prognosis in patients with cancer. Several authors showed that levels of CECs in patients parallel the tumour burden and response to therapy [6–12]. Indeed, the number of circulating tumour cells before treatment was recently found to be an independent predictor of progression-free and overall survival in patients with metastatic breast cancer [13]. CECs can be detected in 0–72% of patients with prostate cancer that is clinically organ-confined and in 25–100% of patients with distant metastatic disease. The presence of CECs at the time of primary therapy has also been associated with early disease failure and poor long-term outcome [14,15]. Various groups also showed that the presence of CECs measured by reverse transcriptase (RT)-PCR for PSA in patients with HRPC receiving cytotoxic chemotherapy correlated with survival outcome [16–20]. Positive RT-PCR for PSA is an independent prognostic factor for survival in men with HRPC [21]. Halabi *et al.* [22] confirmed that RT-PCR for PSA is a statistically significant predictor of overall survival for patients treated once with previous hormonal therapy.

RT-PCR for CECs has several limitations; the lack of specificity coupled with the lack of standardization of RT-PCR techniques has prevented this test from achieving widespread use. By contrast, fluorescence-activated cell sorting (FACS) analysis allows the detection of antigens in a heterogeneous mixture of cells, and offers several advantages over immunohistochemistry and RT-PCR. Cell sorting is easy to do and enables a high throughput of samples, quantification of results, and isolation of subpopulations of cells. The feasibility of using FACS assays for detecting micrometastases was reported in several cancers [12,13,20,23]. Compared with normal individuals there are significantly more CECs identified by FACS analysis in patients with prostate cancer. Also, the presence of CECs in patients with advanced prostate cancer appears to correlate with survival [24–27]. Unfortunately, limited sample sizes and the lack of clinical correlation make these results insufficient to assess the true clinical utility of this test. We report the results of a retrospective pilot analysis that evaluated patients with HRPC undergoing cytotoxic therapy, to determine the utility and feasibility of FACS analysis for detecting CECs, their change over time, and to assess whether or not the presence and number of CECs identified by FACS analysis was a predictor of outcome in men with HRPC.

PATIENTS AND METHODS

This was a retrospective study of 41 consecutively treated patients with metastatic HRPC who were starting systemic chemotherapy. All patients had peripheral blood collected before starting systemic cytotoxic chemotherapy with a taxane-based regimen. Subsequently, blood was collected at the start of each cycle of chemotherapy until therapy was discontinued. All 41 patients have had, and subsequently discontinued, second-line hormonal manipulations before entry to the present study. There were no uniform criteria applied for either the discontinuation of second-line hormonal therapy or the subsequent institution of systemic chemotherapy. For patients with measurable disease, progression was defined as a ≥20% increase in the sum of the longest diameter of target lesions or the appearance of one or more new lesions, as for the Response Evaluation Criteria in Solid Tumors system [28]. Patients with no measurable disease were required to have a positive bone scan and elevated PSA level. PSA evidence for progressive prostate cancer consisted of a PSA level of ≥5 ng/mL, which had risen above the minimum of the nadir and baseline on at least two successive occasions, at least 2 weeks apart. Response to therapy was assessed by Consensus Criteria [29]. There were no uniform criteria applied for the minimum or maximum number of peripheral blood collections required while patients were receiving systemic chemotherapy.

For the isolation and enumeration of CECs, blood samples were drawn into 10-mL EDTA-Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) to which a cell preservative was added [30,31]. Samples were maintained at room temperature and processed within 24 h after collection. All FACS analyses were performed at a central laboratory within our institution. For each sample the lymphocyte/monocyte fractions were separated using Ficoll-Hypaque density-gradient centrifugation. A positive-selection pre-enrichment step was used, by incubating the lymphocyte/monocyte fractions of each sample with ferrofluid particles coated with MJ37 (an anti-epithelial surface antigen encoded by the EGP2 or GA-733-2 gene, EpCAM) monoclonal antibody. The anti-EpCAM (EBA-1) antibody that recognises epitopes different from MJ37 was also added. The sample tube was then subjected to a magnetic field for 45 min in a magnetic separator and the sample blood aspirated from the tube. The sample tube was removed from the magnet and cells remaining in the tube were resuspended in 2 mL of cell buffer. The re-suspended cells were transferred to one 12 × 75 mm polystyrene tube and subjected to magnetic separation for 5 min. The fluid material in the tube was aspirated and the pellet of cells was re-suspended in 150 μL of cell buffer. The antibody CD45 PerCP-Cy5.5 and a nucleic acid dye (ProCOUNT, Becton Dickinson) were added (20 μL). Fluorescently labelled monoclonal antibodies specific for leukocytes (CD45 PerCP-Cy5.5) and ECs (MJ-37 and EBA-1) are used to distinguish ECs from leukocytes. Samples were incubated in the dark for 15 min, and then fixed by adding 350 μL of 1% paraformaldehyde. Subsequently, samples were transferred to a TruCOUNT tube (Becton Dickinson) and then run on a FACS Calibur (Becton Dickinson) with four-colour option, until 35 000 bead events were acquired. Each sample was acquired with a threshold on both EpCAM (EBA-1) and nucleic acid dye (ProCOUNT). Circulating tumour cells were defined as nucleated cells, which are

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Characteristic (n in sample)	Value
Median (range) age, years (40)	70.1 (44–89)
Median (range) initial PSA level, ng/mL (40)	50.2 (0.9–3019)
n (%) with PSA level of:	
<10.0	10 (25)
10.0–100.0	17 (42)
>100.0	13 (33)
Mean (SD) ALP, IU/L	184.8 (211.8)
Median (range)	111.0 (58.0–1160)
Mean (SD) haemoglobin, g/dL (40)	12.7 (2.0)
Median (range)	12.7 (9.1–18.4)
n (%) with <12.0	11 (28)
Mean (SD) LDH, U/L (40)	174.4 (64.1)
Median (range)	152.5 (129–470)
ECOG performance status, n (%)	
0	26 (64)
1	10 (24)
2	5 (12)
Gleason sum, n (%)	
5–6	7 (17)
7	21 (51)
8–9	13 (32)
No. of previous systemic therapies	
0	1 (2)
1	15 (37)
2–3	21 (51)
4–5	4 (10)
Median blood volume/sample, mL	20
Mean (SD, range) volume sampled	128.1 (197.5, 0–1005)
Median (range) CECs/mL	1.8 (0–55.8)
Mean (SD)	6.97 (10.66)
N (%) with CECs/mL of	
0	4 (10)
0.1–5.0	20 (49)
>5.0–15.0	10 (24)
>15.0–30.0	6 (15)
>30.0	1 (2)

TABLE 1
Patient characteristics and CEC counts for the 41 men in the study

simultaneously EpCAM-positive, ProCOUNT-negative and CD45-negative [32,33].

The data analysis was primarily descriptive; each patient's disease characteristics at the time of entering the trial were collected, including PSA level, Gleason score, extension of metastatic disease, details of previous therapy, and laboratory variables. Descriptive statistics were used to characterize the entire patient sample. Subsets were compared using Fisher's exact test for categorical variables, ANOVA methods for continuous variables and the nonparametric Mann–Whitney *U*-test to compare distributions. The association between continuous variables was estimated by the Spearman rank correlation coefficient. The Kaplan–Meier product-limit method was also used to estimate the probability of survival, with the log-rank test used to compare distributions of subsets. Survival was measured from the start of chemotherapy until either death or the date of last contact. Multivariate analyses were done using Cox proportional-hazards model to identify independent predictors of survival. A forward stepwise approach was used, with significance determined by the likelihood-ratio test. Coefficients for significant predictors were tested using the Wald statistic.

RESULTS

FACS analysis data from 41 patients with metastatic HRPC who initiated systemic taxane-based chemotherapy at our institution between 1999 and 2001 were included; their characteristics are summarized in Table 1. All patients had radiographic evidence of metastatic disease in either soft tissue, bone or both (39%, 61% and 22%, respectively). The initial median (range) PSA level for all evaluable patients was 50.2 (0.9–3019) ng/mL; 30% had PSA levels of <20 ng/mL and 80% had an Eastern Cooperative Oncology Group (ECOG) performance status of 0–1. The median (range) alkaline phosphatase (ALP) level was 111 (59–1160) U/L and the median haemoglobin level was 12.7 (9.1–18.4) g/dL. Overall, 51% of patients had a Gleason score of 7, while in 32% it was 8–10. As defined by the consensus criteria, all patients had castrate testosterone levels. More than half of the patients (61%) had received at least two previous systemic therapies that included androgen deprivation, immunotherapy on a clinical trial, and secondary hormonal manoeuvres with agents such as antiandrogens, oestrogens and ketoconazole.

The number of peripheral blood collections in the patients varied; half (51%) had only one collection for FACS analysis just before starting chemotherapy, 49% had more than one collection, and 15% had 7–15 collections. Most patients (66%) had 20 mL of blood collected, and no patient had <9.5 mL collected. When analysed by the volume of blood obtained (<20 vs 20 mL) for the first collection, there was no difference in the number of CECs/mL (data not shown).

There were no CECs in the peripheral blood in only four patients; all four had bone metastases only and their Gleason score was 7 in two and 8 in two. There were no significant differences between this small subset and the entire cohort. Overall, 49% of patients had 0.1–5.0, 24% had >5–15, 15% had >15–30 and 2% had >30 CECs/mL.

Among all patients the number of CECs/mL obtained at the time of first collection was significantly correlated with PSA level, age (inversely) and ALP levels, with a Spearman rank correlation, r, of 0.53 ($P < 0.001$), –0.33 ($P = 0.04$) and 0.38 ($P = 0.02$), respectively. At the time of the first collection the association was strongest between the number of CECs/mL and PSA level ($P = 0.01$). If a patient had a PSA level of <20 ng/mL, then 83% also had <1.8 CECs/mL (the median). There was more variability in range for the CECs/mL if the patient had a PSA level of >20 ng/mL but most (61%) had >1.8 CECs/mL. The decreasing concentration of CECs with increasing age

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reflects that those patients aged <65 years (the lower age quartile) more often had more than the median value of 1.8 CECs/mL (67%), whereas those aged ≥75 years usually had fewer than the median (67%). Of all patients, 80% with ALP levels of >200 U/L (the upper quartile) had >1.8 CECs/mL ($P = 0.02$). By contrast, patients with ALP levels of <110 U/L (the median) were more likely to have <1.8 CECs/mL (65%), resulting in the increasing correlation. For the first collection there was no association between the concentration of CECs and lactate dehydrogenase (LDH), haemoglobin, ECOG performance status or the number of previous therapies. Using the overall median (1.8 CECs/mL) to dichotomize the patients, those with ≤1.8 CECs/mL had significantly longer survival than those with >1.8 CECs/mL. The median survival of patients with metastatic HRPC with >1.8 CECs/mL was 13 months; that for patients with ≤1.8 CECs/mL has not been reached ($P = 0.02$; Fig. 1). Moreover, there were no associations between changes in serum PSA level, serum ALP and the number of CECs/mL with disease response while on therapy. Nevertheless, when several measurements were available, there were often similar patterns over time for CECs/mL, PSA and ALP levels; Fig. 2 shows an example of this relationship.

Additional univariate analyses indicated that having a PSA level of <20 ng/mL, ALP of ≤110 U/L, a Gleason score of ≤7 or having had only one previous therapy resulted in a more favourable survival outcome ($P = 0.01$, 0.03, 0.05 and 0.02, respectively). Multivariate analyses using a Cox proportional-hazards model were used to identify significant independent predictors of survival from among those significant factors determined by univariate methods. This included CECs/mL (≤1.8 vs >1.8), PSA and ALP levels, Gleason score (≤7 vs 8–10) and the number of previous therapies (1 vs >1). Both CECs/mL and the number of previous therapies were independent predictors of survival (likelihood-ratio test, $P = 0.02$ for each factor; Table 2). The median survival for all patients was 18.4 months; 19 of the 41 patients died, all within 20 months of starting chemotherapy, and 10 survived beyond that time for up to 65 months from diagnosis.

FIG. 1. Overall survival vs CECs/mL in patients with HRPC.

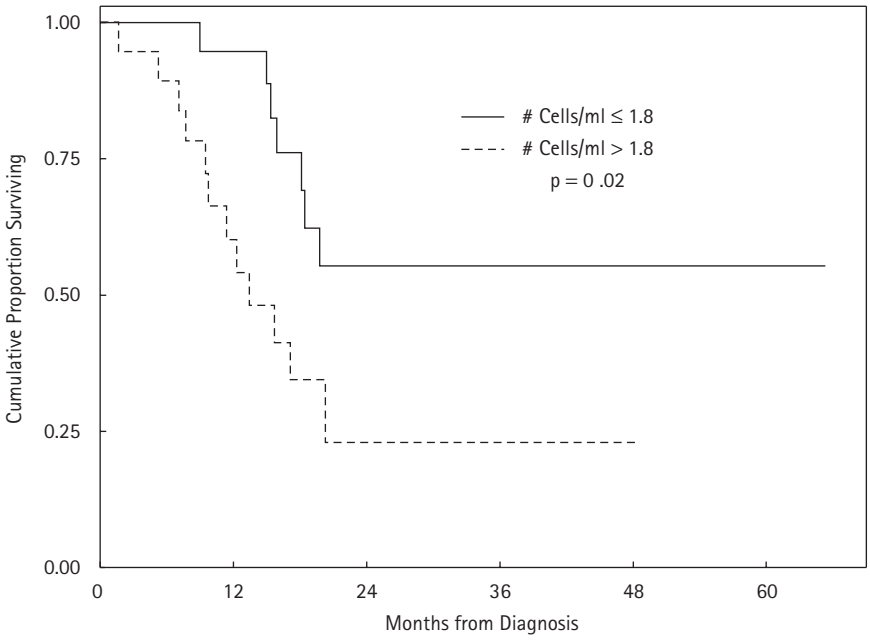
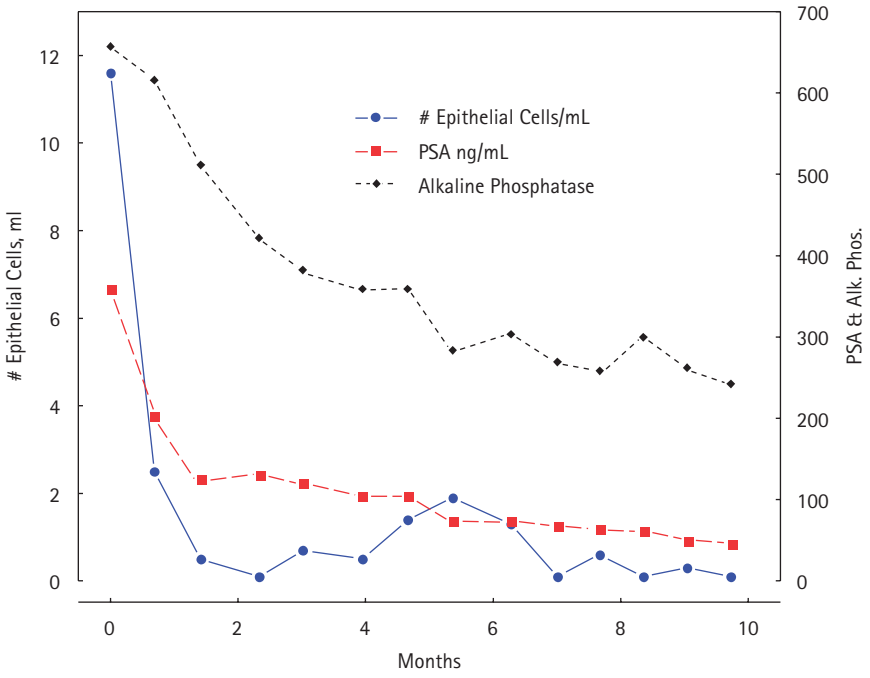


FIG. 2. The relationship between PSA and ALP levels, and CECs in patients with HRPC undergoing palliative systemic cytotoxic chemotherapy. CECs, PSA and ALP levels were recorded at different times during the patient's chemotherapy treatment.



DISCUSSION

This retrospective analysis evaluated the feasibility of using FACS analysis for detecting CECs in patients with HRPC; we also evaluated the correlation between the level of CECs and other clinical variables, e.g. PSA, ALP, LDH, and haemoglobin, all clinical features previously shown to affect the outcome in such patients.

Although the analysis was limited by being retrospective and including relatively few patients, CECs were present in the vast majority of the patients. There was no reference point to relate the time of collection

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TABLE 2 Univariate and multivariate analysis; predictors of survival

Variable	Univariate; log-rank test P	Multivariate; independent predictors of survival		
		Likelihood ratio, P	Hazard ratio (95% CI)	Wald, P
PSA, <20 vs ≥20	0.01			
CEC/mL, ≤1.8 vs >1.8	0.02	0.02	3.2 (1.2–8.3)	0.02
Previous therapy, 1 vs >1	0.02	0.02	3.3 (1.1–9.4)	0.03
ALP, ≤110 vs >110	0.03			
Gleason score, ≤7 vs 8–10	0.05			

with the course of disease, and hence any of the differences noted in this analysis only reflect the data at one point in time and not necessarily a common point for all patients. Therefore, these results require validation in a prospective trial, and cannot be universally applied to all patients with HRPC.

In the present analysis there were very strong correlations between the concentration of CECs, and serum PSA and ALP levels; hence, >1.8 CECs/mL, a serum PSA level of ≥20 ng/mL and serum ALP levels of >110 U/L (the median values) were each strong predictors of a poorer outcome ($P = 0.02$, 0.01 and 0.03, respectively). Unfortunately, this limited study could not define an association between changes in serum PSA and ALP levels, and number of CECs/mL, with disease response. However, it was suggestive that the pattern of CECs/mL measured over time appeared to mirror the PSA pattern (with an increase or plateau) in an individual patient while on chemotherapy. Also, when several measurements were available, there were often similar patterns over time for CECs/mL and ALP levels. This reflects the correlation between these factors that was identified at the initial collection, and might suggest that in addition to clinical symptoms, serum PSA level, and imaging studies, CECs could potentially be used for predicting and assessing the response to systemic chemotherapy in patients with HRPC.

Similar to our data, Moreno *et al.* [34] reported their experience using FACS analysis for evaluating CECs in patients with advanced prostate cancer. Among their 26 patients with HRPC, the presence of ≥5 CECs/7.5 mL of blood was a strong predictor for survival outcome (hazard ratio 7.18, $P = 0.002$). After a multivariate Cox analysis the presence of CECs was of borderline significance in a model for predicting the survival in patients with HRPC (hazard ratio 4.18, $P = 0.056$). Similarly, their study showed that patients with <5 CECs/7.5 mL of blood had a median overall survival time of 2.5 years, compared with 0.5 years in patients with >5 CECs/7.5 mL ($P = 0.003$).

In the present study there were similar associations between the number of CECs/mL and overall survival. We also dichotomized the patient sample based on the overall median number of CECs/mL. With a median follow-up of >36 months, the overall median survival for all metastatic patients with >1.8 CECs/mL was 13 months, and the median for patients with ≤1.8 CECs/mL, overall or with metastases, has not been reached ($P = 0.02$). Our multivariate analysis also indicated that CECs/mL and the number of previous therapies (which probably represents the extent of the disease process, and later stages in treatment) were each independent predictors of survival ($P = 0.02$ for each).

In summary, we showed that in addition to previously described clinical variables, measuring CECs in patients with HRPC can be used as a prognostic tool to predict the outcome. Having more CECs/mL appears to correlate with shorter survival in patients with metastatic HRPC. Our findings, combined with the results from others, suggest that CECs might be relevant and could be used to predict the outcome in patients with HRPC. Future clinical trials with chemotherapy or novel therapeutics in patients with HRPC should consider the prospective collection of peripheral blood for CEC analyses.

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Abbreviations: **HRPC**, hormone-refractory prostate cancer; **CEC**, circulating epithelial cell; **RT**, reverse transcriptase; **FACS**, fluorescence-activated cell sorting; **ALP**, alkaline phosphatase; **ECOG**, Eastern Cooperative Oncology Group; **LDH**, lactate dehydrogenase.

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Activity of Second-Line Chemotherapy in Docetaxel-Refractory Hormone-Refractory Prostate Cancer Patients

Randomized Phase 2 Study of Ixabepilone or Mitoxantrone and Prednisone

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BACKGROUND. This randomized, noncomparative, multicenter, clinical trial evaluated ixabepilone or mitoxantrone/prednisone (MP) as second-line chemotherapy for taxane-refractory, hormone-refractory, prostate cancer (HRPC).

METHODS. Patients with HRPC that progressed during or within 60 days of cessation of taxane chemotherapy were randomly selected with equal probability to ixabepilone 35 mg/m^2 intravenously every 3 weeks, or mitoxantrone 14 mg/m^2 intravenously every 3 weeks and prednisone 5 mg orally twice daily. Treatment continued until progression or toxicity; crossover was allowed.

RESULTS. Forty-one patients were accrued to each arm of the study. The median number of cycles administered for each arm was 3. Median survival from protocol entry was 10.4 months with ixabepilone and 9.8 months with MP. Prostate-specific antigen (PSA) declines of $\geq$50% were observed in 17% of ixabepilone (95% CI, 7-32) and 20% of second-line MP patients (95% CI, 9-35). Partial responses were observed in 1 of 24 ixabepilone and in 2 of 21 MP patients with evaluable measurable disease. Median duration of second-line ixabepilone and MP treatment was 2.2 months and 2.3 months, respectively. For third-line crossover treatment, PSA declines of $\geq$50% were observed in 3 of 27 ixabepilone-treated and 4 of 15 MP-treated patients. Prior taxane response was associated with an increased likelihood of second-line ixabepilone or MP response. Low baseline lactate dehydrogenase and absence of visceral metastases independently predicted improved survival. The most common grade 3/4 toxicity associated with second-line treatment was neutropenia (54% of ixabepilone patients and 63% of MP patients).

CONCLUSIONS. Ixabepilone and MP had modest activity as second-line chemotherapy for docetaxel-refractory HRPC. The median survival for the entire cohort treated in this study was 9.8 months. ***Cancer* 2007;110:556–63.** *© 2007 American Cancer Society.*

KEYWORDS: prostate cancer, taxane, hormone, refractory, ixabepilone, mitoxantrone, prednisone, second-line therapy.

Chemotherapy for taxane-refractory, hormone-refractory, prostate cancer (HRPC) is effective at prolonging survival and palliating symptoms. Two large phase 3 studies demonstrated that first-line docetaxel chemotherapy is associated with an improvement in median survival compared with mitoxantrone/prednisone (MP).[1,2]

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Nearly all HRPC patients eventually progress during or after taxane-based treatment. Many patients have a good performance status and wish additional treatment. No standard chemotherapy exists for second-line treatment of patients with HRPC after progression on taxane-based therapies, although the community de facto standard has become MP.

The natural history of taxane-refractory (TR) HRPC has not been prospectively defined. Although second-line chemotherapy trials have been reported in HRPC, these trials are difficult to interpret because of heterogeneity of patient populations. Most importantly, those trials did not restrict enrollment to overtly TR-HRPC.

Resistance to taxanes appears mediated by tubulin mutation and multidrug resistant (MDR) gene overexpression. The epothilones are a new class of nontaxane tubulin polymerization agents whose cytotoxic activity has been linked to stabilization of microtubules, bypassing known taxane-resistant mechanisms.[3,4] Ixabepilone (Bristol-Myers Squibb, New York, NY) is a semisynthetic analog of epothilone B that blocks the mitotic phase of the cell cycle. It is a highly potent cytotoxin, and preclinical data demonstrate noncross-resistance with taxanes. Ixabepilone has demonstrated antitumor activity as first-line chemotherapy in patients with metastatic HRPC.[5,6]

The preclinical data indicating noncross-resistance of ixabepilone with taxanes, the front-line activity of ixabepilone in HRPC, and the lack of prospective data regarding MP as second-line chemotherapy provided the rationale for a randomized, noncomparative, phase 2 study in TR-HRPC. This study randomly assigned patients with TR-HRPC to either single-agent ixabepilone or the perceived community standard, MP.

MATERIALS AND METHODS

Study Design

This study was a multicenter, randomized, noncomparative phase 2 study. Patients were randomly assigned with equal probability to either MP or ixabepilone. The primary endpoint was the frequency of $\geq$50% PSA declines with each second-line regimen. Secondary endpoints included safety, response duration, time to progressive disease, third-line (post-crossover) activity of each regimen, and overall survival.

Eligibility Criteria

All patients had histologically confirmed metastatic prostate cancer. Patients were required to have progressive disease despite castrate testosterone levels and at least 2 cycles of taxane-based chemotherapy, with disease progression documented during or within 60 days of completing taxane-based chemotherapy. For patients with measurable disease, progression was defined by RECIST criteria.[7] For patients without measurable disease, a positive bone scan and elevated PSA greater than 5 ng/mL were required. PSA evidence for progressive prostate cancer was defined by Consensus Criteria.[8]

All patients were required to have an Eastern Cooperative Oncology Group (ECOG) performance status of 0-2 and $\leq$grade 1 neuropathy (Common Toxicity Criteria, version 2.0). Hormonal therapy other than luteinizing hormone-releasing hormone (LHRH) agonists was not allowed within 4 weeks of trial enrollment (6 weeks for bicalutamide or nilutamide). Treatment with a corticosteroid as part of first-line chemotherapy was discontinued over 10–14 days before enrollment. Any radiation therapy or radiopharmaceutical treatment must have been completed more than 4 weeks and 8 weeks before enrollment, respectively. All patients were required to have a cardiac ejection fraction greater than the institutional lower limit of normal. Patients were excluded for significant cardiovascular disease including congestive heart failure (New York Heart Association [NYHA] class III or IV), active angina pectoris, or myocardial infarction within 6 months before enrollment. Patients with known active brain metastases were excluded. Required laboratory values included testosterone $<$50 ng/dL; creatinine $<1.5 \times$ upper limits of normal (ULN) or calculated creatinine clearance $>$40 mL/min; alanine aminotransferase (ALT) and aspartate transaminase (AST) $<3 \times$ ULN; granulocytes $>1500/mm^3$; platelets $\geq 100{,}000/mm^3$; total bilirubin $<1.5 \times$ ULN; and, if no measurable disease, a PSA $\geq$5 ng/mL.

This clinical trial was sponsored by the Cancer Therapy Evaluation Program of the National Cancer Institute and approved by the review boards of each participating institution. All patients provided written informed consent.

Randomization and Treatment Plan

Eligible patients were randomly selected by the coordinating center statistician with equal probability to receive either ixabepilone or MP. Allocation to a treatment arm was concealed until the patient was enrolled. Patients were stratified by performance score (0 vs 1-2) and study site, and they were randomly assigned from within each stratum. Treatment assignment was balanced after every 4 patients within each stratum.

Ixabepilone 35 mg/m^2 was administered intravenously over 3 hours every 21 days. Patients were

premedicated with H1- and H2-blockers before ixabepilone infusion to prevent hypersensitivity reactions related to Cremophor EL diluent (BASF Group, Ludwigshafen, Germany) Corticosteroids were used with subsequent cycles for prior grade 2-4 hypersensitivity reactions to ixabepilone. Mitoxantrone 14 mg/m^2 was administered intravenously every 21 days with prednisone 5 mg orally twice daily. Treatment for all patients was continued until disease progression or unacceptable toxicity occurred. Myeloid growth factors were administered according to American Society for Clinical Oncology (ASCO) guidelines.[9] Patients underwent imaging with chest s-ray, bone scan, and computed tomography (CT) or magnetic resonance imaging (MRI) of the abdomen and pelvis at baseline and after every 3 cycles. Electrocardiogram and multiple gated-acquisition (MUGA) scan or echocardiogram were obtained at baseline and repeated every 3 cycles for MP patients. Imaging studies were obtained at the time of crossover.

Dose Modifications

Dose modifications were made according to maximal toxicity. Doses were reduced for Day 1 neutrophil count $<1500/m^3$ or platelet count $<100,000/m^3$, $\geq$grade 3 nonhematologic toxicity, grade 4 neutropenia lasting for more than 7 days, grade 4 neutropenia and fever, and nadir platelet count $<25,000$. Ixabepilone dose was reduced by 5 mg/m^2, and mitoxantrone dose was reduced by 2 mg/m^2 for each dose reduction. Grade 2 neurotoxicity of any duration and grade 3 neurotoxicity lasting $\leq$7 days required dose reduction. Recurrent grade 3 neurotoxicity, grade 3 neurotoxicity of $>$7 days duration, or grade 4 neurotoxicity required discontinuation of treatment. Patients were removed from protocol therapy for a treatment delay greater than 3 weeks or recurrence of the same grade $\geq$3 toxicities despite 2 dose reductions.

Crossover Therapy

Patients who progressed after at least 2 cycles of protocol treatment or who stopped treatment for toxicity or other medical reasons were eligible to receive the alternate treatment. For patients initially treated with MP, prednisone was tapered over 10–14 days before starting ixabepilone.

Statistical Considerations

This was a noncomparative randomized phase 2 study to assess safety and efficacy of 2 treatment regimens, ixabepilone and MP, as second-line therapy for metastatic TR-HRPC patients. The primary endpoint was the frequency of PSA declines $\geq$50% with second-line therapy, confirmed with 2 consecutive measurements. Response to therapy was determined for each patient by using PSA declines for nonmeasurable disease, and RECIST criteria for measurable disease, bone scans, and nontarget lesions.[7,8] For each treatment arm, a $\geq$50% PSA decline in at least 25% of patients was considered promising and worthy of further investigation. Accrual of 40 patients to each treatment arm was sufficient to detect a 25% response proportion compared with a null hypothesis of $\leq$10%. A statistical level of significance of 0.04 for a directional test and power of 0.82 was assumed to test this hypothesis. Secondary endpoints included response duration, time to PSA progression, overall survival, frequency of toxicity, and frequency of response to third-line (crossover) treatment.

Comparability of the 2 treatment subsets was evaluated by using Fisher exact test for categorical variables (eg, Gleason score), Student *t* test for continuous variables (eg, lactate dehydrogenase [LDH]), and the Mann-Whitney test for distributions (eg, PSA). The effect of prior taxane response on second-line treatment response was analyzed by using the Mantel-Haenszel tests of association and homogeneity stratified by the second-line therapy.[10] Duration of time to progression and overall survival were calculated from the start of second-line therapy with the Kaplan-Meier product-limit method.[11] Comparisons of a difference in distributions between subsets were performed by using the log-rank test.[12] Cox proportional hazard model was used to identify independent disease features of overall survival for the entire sample.[13] Variables predictive of overall survival based on the log-rank test were considered in building a model. A forward stepwise approach was used with the likelihood ratio test to determine significant independent predictors of survival.

RESULTS

Patient Characteristics and Disposition

Between February 2003 and June 2005, 86 patients were entered at 6 participating centers. Four patients who never started protocol therapy were not included in the analysis, thus 82 patients were evaluable. Forty-one patients were randomly assigned to each treatment arm (Fig. 1). Patient baseline characteristics are detailed in Table 1. Both arms were balanced. All patients who received any protocol chemotherapy were included in evaluations of response and toxicity.

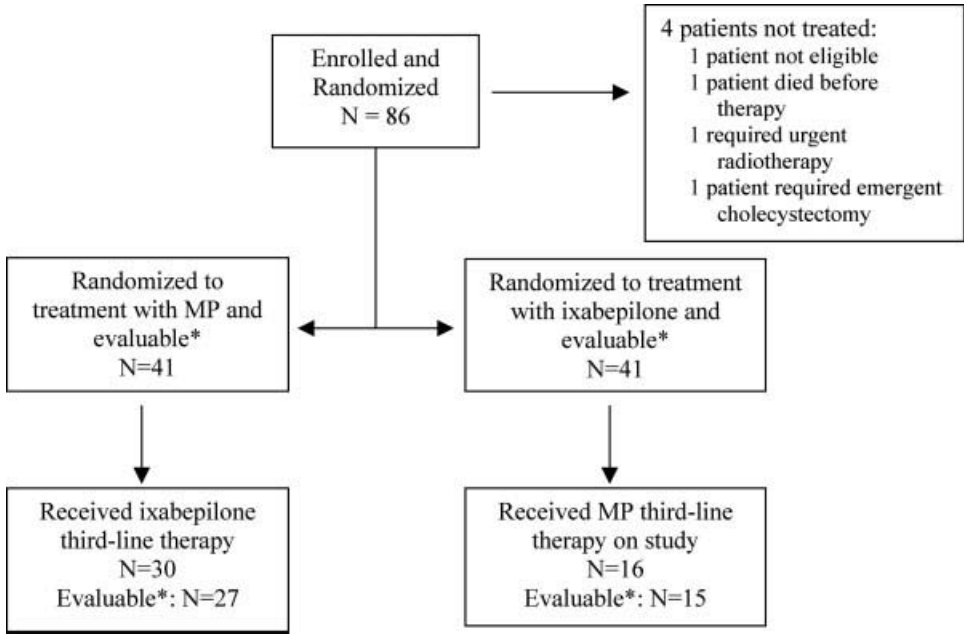

FIGURE 1. Patient Disposition. *Received at least 2 cycles of therapy.

TABLE 1
Baseline Patient Characteristics

2nd Line treatment	Ixabepilone n = 41	MP n = 41
Median age, y (range)	66.5 (51–87)	69 (52–84)
ECOG PS		
0	15 (37%)	15 (37%)
1–2	26 (63%)	26 (63%)
Prior therapy		
Radiation (RT)	10 (24%)	7 (17%)
Prostatectomy (RP)	16 (39%)	15 (37%)
RP+RT	2 (5%)	5 (12%)
Other	13 (32%)	14 (34%)
Median PSA, ng/mL (range)	141 (4–17,995)	113 (7–1587)
Gleason score	n = 37	n = 38
Range	5–10	5–10
5–6	14%	11%
7	32%	18%
8–10	54%	71%
Median LDH, IU/L (range)	266 (103–2291)	273 (101–3065)
Median alkaline phosphatase, U/L (range)	126 (58–1432)	156 (45–664)
Median hemoglobin, g/dL (range)	11.7 (8.8–14.0)	12.2 (8.9–14.7)
Mean No. prior taxane chemotherapy cycles (range)	5.6 (2–25)	6.8 (2–17)
Prior chemotherapy		
Docetaxel-based	18 (45%)	18 (47%)
Docetaxel/estramustine-based	22 (55%)	20 (53%)

Second-Line Study Treatment

A median of 3 cycles of ixabepilone (range, 1 to 22 cycles) and 3 cycles of MP (range, 1 to 12 cycles) were administered as second-line treatment. Thirty-two percent of ixabepilone patients and 27% of MP patients received at least 5 cycles of therapy. Treatment with ixabepilone was discontinued in 7 patients for toxicity, 1 for withdrawal of consent, and 33 patients for disease progression (23 for PSA progression, 6 for objective progression, 1 for both PSA and objective progression, and 4 for clinical and/or symptomatic progression that required additional

TABLE 2
Response to Second-line Therapy

	2nd-Line Ixabepilone no. (%)	2nd-Line MP no. (%)
Evaluable patients	41	41
Confirmed PSA decline ≥50%, 95% CI	7 (17, 7–32)	8 (20, 9–35)
Unconfirmed PSA decline ≥50%	1 (2)	—
Objective disease responses		
Measurable disease	30	23
Evaluable patients*	24	21
Partial response (RECIST)	1	2

* Received at least 2 cycles.

therapy). Treatment with MP was discontinued in 4 patients for toxicity and in 36 patients for disease progression (28 for PSA progression, 6 for objective progression, 2 for both PSA and objective progression). One MP patient died on study of unrelated causes.

Response

Of 41 patients treated with second-line ixabepilone, 7 had a confirmed ≥50% PSA decline (17%; 95% CI, 7-32; Table 2). One additional patient had an unconfirmed ≥50% PSA decline. The median time to a ≥50% PSA decline was 6 weeks (range, 3–14 weeks). Twenty-four patients treated with at least 2 cycles of second-line ixabepilone had measurable disease, and, of these, 1 (4%) patient had an objective partial response in addition to a PSA response. The median time to PSA progression on ixabepilone was 2.2 months, and the median duration of response was 3.8 months (range, 2.8–22.3 months). Three confirmed responders discontinued treatment for toxicity (motor neuropathy, atrial arrhythmia, and grade 2 infusion-site reaction), and 4 confirmed responders discontinued because of progressive disease.

Of the 41 patients treated with second-line MP, 8 had a confirmed ≥50% PSA decline (20%; 95% CI, 9-35; Table 2). For responders, the median time to a ≥50% PSA decline was 7 weeks (range, 3–19 weeks). Twenty-one patients treated with at least 2 cycles of second-line MP had measurable disease, and, of these, 2 (10%) patients had an objective partial response, 1 of whom also had a PSA response. The median time to PSA progression on MP was 2.3 months, and the median duration of PSA response for responders was 5.9 months (range, 2.7–8.2 months). Three responders discontinued treatment because of toxicity (minor decreases in cardiac ejection fraction did not meet criteria for an adverse event according to National Cancer Institute's Common Toxicity Criteria v2.0 in 2 patients; thrombocytopenia occurred in 1 patient), 4 discontinued for progressive disease, and 1 died without disease progression.

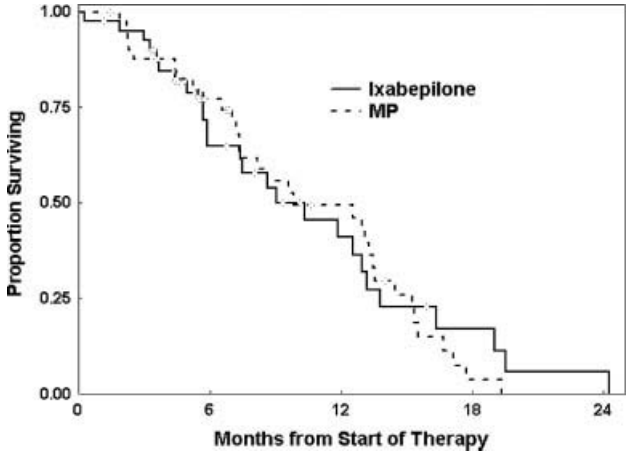

FIGURE 2. Overall survival.

An exploratory analysis of the impact of initial response to front-line taxane-based therapy on response to second-line therapy was performed. Stratified by second-line treatment, there was a significantly greater response to second-line therapy among patients who previously responded to taxane therapy (Mantel-Haenszel test: $P = .0004$).[10] The association was similar for both second-line treatment groups (test of homogeneity: $P = 0.87$). Among patients with a prior PSA response to taxane chemotherapy, 36% (5 of 14; 95% CI, 13-65) responded to ixabepilone and 35% (7 of 20; 95% CI, 5-59) responded to MP. In patients without prior PSA response to taxane-chemotherapy, 4% (1 of 26; 95% CI, 0-20) of patients responded to ixabepilone, and 5% (1 of 21; 95% CI, 0-24) responded to MP.

Survival

Evaluation of survival by treatment is complicated by the finding that 56% of patients received the alternate therapy on crossover. However, the median survival for each arm was 10.4 months for ixabepilone and 9.8 months for MP. (Fig. 2) The median overall survival for the entire study was 9.8 months., and did not show differences based on prior taxane response.

Potential disease features predictive of survival from the start of second-line therapy were evaluated in patients enrolled on this study in an exploratory analysis. When the entire study sample was dichotomized at the median baseline value, a significantly prolonged survival was observed for decreased LDH (≤270 vs >270), decreased alkaline phosphatase (≤130 vs >130) and increased hemoglobin (≤12 vs >12) ($P = .007$, .003, and .01, respectively).

TABLE 3
Maximal Grade 3-4 Hematologic Toxicity

	Ixabepilone		MP	
	2nd-Line, n = 41	3rd-Line, n = 29	2nd-Line, n = 41	3rd-Line, n = 16
	No. (%)	No. (%)	No. (%)	No. (%)
Anemia	4 (10)	2 (7)	1 (2)	—
Neutropenia	22 (54)	10 (33)	26 (63)	10 (63)
Febrile neutropenia	2 (5)*	2 (7)	4 (10)	—
Thrombocytopenia	3 (7)	3 (10)	1 (2)	1 (6)

* 1 patient died of neutropenic sepsis.

TABLE 4
Maximal Grade 3-4 Treatment-Related Non-Hematologic Toxicity

	Ixabepilone				MP			
	2nd-Line, n = 41		3rd-Line, n = 30		2nd-Line, n = 41		3rd-Line, n = 16	
Grade	3	4	3	4	3	4	3	4
GI								
Nausea/vomiting	2		1				2	
Anorexia	2							
Stomatis/pharyngitis	3		1					
Diarrhea			1					
Constipation					1			
Dehydration	1		3					
Hepatic	2		1		4			
Hypotension			3					
Fatigue	1	1	4					
Muscle weakness	2		2					
Renal		1						
Neurologic								
Motor neuropathy	1		2					
Sensory neuropathy			1					
CNS ischemia		1						
Syncope	1							
Lightheadedness	1		1					
Mood alteration	1							
Elevated PT	3		1		2			
Metabolic								
Hypophosphatemia	1		3					
Hypoglycemia				1				
Hyperuricemia		1						
Hypercalcemia		1	1					
Hypokalemia			1					
Hypersensitivity		1	1					

The following grade 3 toxicities occurred with second-line ixabepilone in 1 patient: thrombosis, atrial arrhythmia, urinary obstruction, and chest pain.

The 3 laboratory parameters were highly correlated ($P < .002$ for all pairwise comparisons). Patients without visceral disease also achieved a significantly longer survival ($P = .02$). Categorized LDH ($\leq$270 vs >270) was highly associated with visceral disease ($P = .005$). There was no difference in survival due to baseline performance score, PSA, or Gleason score. When the 4 individual parameters significant to predicting survival were considered simultaneously by using Cox proportional hazard model, a decreased LDH and absence of visceral metastases emerged as significant independent predictors of prolonged survival (likelihood ratio test, $P = .0003$, .04, respectively).

Toxicity

Grade 3 or 4 neutropenia occurred in 54% and 63% of patients treated with second-line ixabepilone and MP, respectively (Table 3). Febrile neutropenia and neutropenic infection occurred in 4 patients treated with second-line MP and 3 patients treated with second-line ixabepilone (including 1 patient who died from neutropenic sepsis). Treatment-related nonhematologic toxicities observed in $\geq$5% of patients treated with second-line ixabepilone included anorexia, stomatitis, fatigue, muscle weakness, and prolonged prothrombin time (Table 4). Treatment-related nonhematologic toxicity observed in $\geq$5% of patients treated with second-line MP included prolonged prothrombin times and liver function abnormalities. Dose reduction or delay were required in 20 of 41 (49%) patients treated with second-line ixabepilone and 10 of 41 (24%) patients treated with second-line MP.

Crossover Therapy

Sixteen of 41 (39%) patients on second-line ixabepilone crossed over to MP treatment. Of the 25 patients who did not cross over to MP, 8 withdrew consent, 2 died, and 14 experienced clinically significant disease progression and/or treatment-related toxicity such that they did not cross over. Four of 15 evaluable patients who received third-line MP achieved a confirmed $\geq$50% PSA decline (27%; 95% CI, 8-55; Table 5). One of 9 (11%) patients with measurable disease and at least 2 cycles of therapy demonstrated an objective response to third-line MP in addition to a PSA response.

Thirty of 41 (73%) patients on second-line MP crossed over to ixabepilone therapy. Of the 11 patients who did not cross over to ixabepilone, 2 withdrew consent, 1 died, 1 was not eligible to continue on study because of decreased clinical status, and 7 patients experienced clinically significant disease progression and/or treatment-related toxicity such that they did not cross over. Three of 27 (11%; 95% CI, 2-29)evaluable patients achieved a confirmed $\geq$50% PSA decline to third-line ixabepilone. One of 14 (7%) patients with measurable disease and at least

TABLE 5
Response to Crossover Therapy

	3rd-Line MP, n = 16	3rd-Line ixabepilone, n = 30
PSA responses	**No. (%)**	**No. (%)**
Evaluable patients*	15	27
Confirmed PSA decline ≥50%, 95% CI	4 (27, 8-55)	3 (11, 2-29)
Unconfirmed PSA decline ≥50%	—	1 (4)
Objective disease responses		
Measurable disease	11	15
Evaluable patients*	9	14
Partial response (RECIST)	1	1

* Received at least 2 cycles.

2 cycles of therapy demonstrated both an objective and a PSA response.

None of the patients who achieved a PSA response to third-line therapy demonstrated a PSA response to second-line treatment. None of the patients who responded to third-line ixabepilone and only 1 patient who responded to third-line MP had achieved a previous response to front-line taxane chemotherapy.

DISCUSSION

This study evaluated second-line chemotherapy in TR-HRPC patients to address the question of clinical cross-resistance between taxanes, epothilones, and mitoxantrone, as well as to explore the natural history of chemotherapy-refractory HRPC. MP is the de facto community standard second-line chemotherapy for HRPC in the absence of prospective data in this setting. Therefore, determining the activity of second-line MP is important not only to understand the usefulness of this regimen as second-line chemotherapy but also to define its activity as a control arm for future second-line clinical trials. Encouraging preclinical activity in taxane-resistant model systems and substantial activity seen in front-line HRPC chemotherapy support the testing of ixabepilone in the second-line setting.

The median survival for patients with TR-HRPC has not been prospectively evaluated. In the present multicenter study, the median survival of all patients was 9.8 months from the initiation of second-line chemotherapy. As study treatments demonstrated only modest activity in this setting, this value provides a useful estimate of survival as a baseline for development of future clinical trials in this patient population.

Treatment of TR-HRPC with MP or ixabepilone demonstrated only modest activity. The PSA response proportions for MP and ixabepilone were 20% and 17%, respectively. Objective responses were infrequent (≤10% each arm). Although this study was not designed to compare the 2 regimens, the levels of activity in this study appear similar between the 2 arms. The anticancer activity of ixabepilone as measured by PSA declines and objective tumor responses contrasts with results of chemotherapy-naive HRPC trials with this drug. Although 17% of patients did experience PSA responses to ixabepilone in this study, this level of activity is not sufficient to justify further evaluation of ixabepilone in this dose and schedule as single-agent second-line HRPC chemotherapy.

Although patients were required to have progressive disease during or shortly after stopping taxane chemotherapy, 35% of ixabepilone and 49% of MP patients previously experienced a ≥50% PSA decline to first-line taxane therapy. A retrospective analysis demonstrated that patients who experienced a PSA response to prior therapy were 7-fold to 8-fold more likely to respond to either second-line regimen. On the basis of these findings, future randomized studies should stratify patients for best response to prior therapy. In addition, patients who never responded to taxane-based therapy are unlikely to respond to ixabepilone or MP, and investigational therapy should be considered. In an exploratory analysis, elevated LDH and the presence of visceral metastases appear to be independent prognostic indicators of poor overall survival in the second-line setting. These indicators should be investigated further in future second-line chemotherapy studies.

The predominant toxicities seen were hematologic in nature. MP was well tolerated, with only 1 episode of neutropenic infection. Ixabepilone treatment resulted in 1 treatment-related death from neutropenic sepsis during Cycle 1. Although nonhematologic toxicities were seen with ixabepilone, none were observed with high frequency, and no single toxicity predominated. Low rates of neurotoxicity seen in this study compared with other trials of ixabepilone may in part be explained by the requirement that all patients enrolled were required to have grade ≤1 neuropathy after taxane chemotherapy. This requirement may have selected a population less susceptible to neuropathy.

Previously, the noncross-resistance of taxanes and ixabepilone was reported in a retrospective analysis of patients treated on a randomized phase 2 trial of first-line ixabepilone with or without estramustine.[14] In that analysis of 49 patients, 51% of patients treated with second-line taxane achieved a ≥50% PSA decline. The results of the current study suggest there may be a sequence-dependent effect of epothilone and that taxane therapy that may be responsible

for the lower level of activity seen with second-line ixabepilone.

In the present study, some patients who experienced disease progression on either MP or ixabepilone and crossed over to the third-line therapy achieved third-line PSA responses. In fact, none of the patients who responded to their third-line treatment responded to their second-line therapy. This implies some non-cross-resistance between the 2 regimens.

Although substantial progress in treating HRPC has been achieved with the introduction of effective first-line chemotherapy, the identification of new agents with high activity in front-line and TR-HRPC patients remains a priority. Median survival of patients with TR-prostate cancer from the start of second-line chemotherapy remains short. Both novel biologic agents as well as novel chemotherapies must continue to be investigated to improve survival in this patient population. Stratification by prior treatment response should be incorporated into future randomized clinical trials.

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ORIGINAL REPORT

Phase I Study of Ixabepilone, Mitoxantrone, and Prednisone in Patients With Metastatic Castration-Resistant Prostate Cancer Previously Treated With Docetaxel-Based Therapy: A Study of the Department of Defense Prostate Cancer Clinical Trials Consortium

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Clinical Trials repository link available on JCO.org.

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The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

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ABSTRACT

Purpose
Mitoxantrone plus prednisone and ixabepilone each have modest activity as second-line chemotherapy in docetaxel-refractory castration-resistant prostate cancer (CRPC) patients. Clinical noncrossresistance was previously observed.

Patients and Methods
Metastatic CRPC patients progressing during or after taxane-based chemotherapy enrolled in a phase I multicenter study of ixabepilone and mitoxantrone administered every 21 days along with prednisone. Ixabepilone and mitoxantrone doses were alternately escalated in a standard 3 + 3 design. Patients were evaluated for toxicity and disease response. Dose-limiting toxicities (DLTs) were defined as treatment related, occurring during cycle 1, and included grade 4 prolonged or febrile neutropenia, thrombocytopenia (grade 4 or grade 3 with bleeding), or ≥ grade 3 nonhematologic toxicity.

Results
Thirty-six patients were treated; 59% of patients experienced grade 3/4 neutropenia. DLTs included grade 3 diarrhea (n = 1), prolonged grade 4 neutropenia (n = 4), and grade 5 neutropenic infection (n = 1). Due to prolonged neutropenia, the highest dose levels were repeated with pegfilgrastim on day 2 of each cycle. The maximum tolerated dose in combination with pegfilgrastim was not exceeded. The recommended phase II dose is mitoxantrone 12 mg/m^2 and ixabepilone 35 mg/m^2 every 21 days, pegfilgrastim 6 mg subcutaneously day 2, and continuous prednisone 5 mg twice per day. Thirty-one percent of patients have experienced ≥ 50% prostate-specific antigen (PSA) declines, and two experienced objective responses. Of 21 patients treated with mitoxantrone 12 mg/m^2 plus ixabepilone ≥ 30 mg/m^2, nine (43%) experienced ≥ 50% PSA declines (95% CI, 22% to 66%).

Conclusion
These results suggest that the combination of ixabepilone and mitoxantrone is feasible and active in CRPC and requires dosing with pegfilgrastim.

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INTRODUCTION

Docetaxel improves survival for patients with metastatic castration-resistant prostate cancer (CRPC).[1,2] However, the median progression-free survival with docetaxel is approximately 6 months, and many patients with disease progression after docetaxel treatment remain in otherwise reasonable health with a good performance status.[1] No standard therapy exists for treatment of CRPC patients with progression following docetaxel therapy.

Ixabepilone and mitoxantrone are two agents that may have utility in CRPC patients whose disease has progressed after docetaxel. Ixabepilone (Ixempra; Bristol Myers-Squib, New York, NY) is a semi-synthetic epothilone analog recently approved by the US Food and Drug Administration for the treatment of chemotherapy-refractory metastatic breast cancer. Ixabepilone has demonstrated evidence of activity in taxane-resistant cell lines, as well as substantial activity in the first-line treatment of CRPC.[3,4] Similarly, mitoxantrone

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(plus a corticosteroid) has demonstrated palliative activity in first-line therapy for CRPC.[5,6] We have previously reported the activity of ixabepilone or mitoxantrone and prednisone (MP) in patients with taxane-refractory CRPC.[7] Both regimens demonstrated modest activity (ixabepilone ≥ 50% prostate-specific antigen [PSA] decline in 17% of patients, MP ≥ 50% PSA decline in 20% of patients). On planned cross-over to the other agent, 11% and 27% of patients demonstrated third-line PSA responses to ixabepilone and MP, respectively, suggesting some clinical noncrossresistance between the two regimens. This has provided the rationale to test the efficacy of ixabepilone administered in combination with MP to patients with disease progression during or after docetaxel-based first-line chemotherapy. While toxicities of these two regimens are somewhat nonoverlapping, concern regarding the use of two potentially myelosuppressive regimens in elderly patients with heavy pretreatment and potential bone marrow involvement mandated cautious dose escalation.

PATIENTS AND METHODS

Study Design

This study was a multicenter, single arm, phase I dose escalation study testing the safety of ixabepilone and MP in CRPC patients with progression during or after prior docetaxel-based chemotherapy. The study was designed to alternately escalate ixabepilone and mitoxantrone for each subsequent dose cohort. A total of six dose combinations were planned as displayed in Table 1. The standard phase I escalation criteria based on the number of DLTs were applied to each dose cohort. The primary end point of the study was to determine the maximum tolerated dose of the combination. Secondary end points included overall safety and frequency of PSA declines and objective responses.

Eligibility Criteria

All patients had histologically confirmed metastatic prostate cancer. Patients were required to have progressive disease despite castrate testosterone levels and at least three cycles of prior taxane-based chemotherapy. Patients were not allowed to have received more than one prior chemotherapy regimen. For patients with measurable disease, progression was defined by Response Evaluation Criteria in Solid Tumors.[8] For patients without measurable disease, a positive bone scan and elevated PSA higher than 5 ng/mL were required. PSA evidence for progressive disease was defined by PSA Working Group 1 Consensus Criteria.[9]

All patients were required to have Eastern Cooperative Oncology Group performance status of 0 to 2, and ≤ grade 1 peripheral neuropathy (National Cancer Institute Common Toxicity Criteria, version 3.0). Hormone therapy other than luteinizing hormone-releasing hormone agonist or a stable dose of corticosteroid from prior treatment was not allowed within 4 weeks of trial enrollment. Any radiotherapy or radiopharmaceutical treatment must have been completed more than 4 weeks and 8 weeks before enrollment, respectively. All patients were required to have a cardiac ejection fraction greater than the lower limit of institutional normal. Patients with significant cardiovascular disease including congestive heart failure (New York Heart Association class III or IV), active angina pectoris, or myocardial infarction within 6 months were excluded. Patients with known active brain metastases were excluded. Required laboratory values included testosterone lower than 50 ng/dL; creatinine ≤ 1.5× upper limits of normal (ULN) or calculated creatinine clearance of 40 mL/min; ALT and AST lower than 2.5× ULN; granulocytes ≥ 2,000/mm^3; platelets ≥ 100,000/mm^3; total bilirubin lower than 1.5× ULN; and, if no bidimensionally measurable disease, PSA ≥ 5 ng/mL. Because ixabepilone is a major CYP3A4 substrate, concurrent use of moderate to strong CYP3A4 inhibitors was strongly discouraged.

This clinical trial was sponsored by the Cancer Therapy Evaluation Program of the National Cancer Institute, conducted through the Department of Defense Prostate Cancer Clinical Trials Consortium, and approved by the institutional review boards of each participating center. All patients provided written informed consent.

Table 1. Dose Escalation Schema

Dose Level	Regimen			
	Mitoxantrone (mg/m^2)	BMS-247550 (ixabepilone; mg/m^2)	Prednisone (mg PO BID)	Pegfilgrastim (mg SC on day 2)
I	8	20	5	—
II	8	25	5	—
III	10	25	5	—
IV	10	30	5	—
V	12	30	5	—
VI	12	35	5	—
Va	12	30	5	6
VIa	12	35	5	6

Abbreviations: PO, orally; BID, twice per day; SC, subcutaneously.

Treatment Plan

The dose escalation schema is depicted in Table 1. Patients were treated every 21 days. Patients were premedicated 1 hour before ixabepilone treatment with oral H1- and H2-blockers to prevent hypersensitivity reactions. For patients who developed grade 2 to 4 hypersensitivity reactions to ixabepilone, corticosteroid premedication was used with subsequent cycles. Patients received mitoxantrone intravenously over 30 minutes, followed by ixabepilone intravenously over 3 hours on day 1 of each cycle. Prednisone was given 5-mg twice daily continuously. Patients on dose levels Va and VIa received prophylactic subcutaneous pegfilgrastim on day 2. Patients had complete blood counts tested on days 8 and 15 of each cycle. Patients were treated until disease progression or unacceptable toxicity. Patients underwent imaging with chest x-ray, bone scan, and computed tomography or magnetic resonance imaging of the abdomen and pelvis at baseline and after every 3 cycles. ECG and either multiple gated-acquisition scan or echocardiogram were obtained at baseline and repeated every 3 cycles.

Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria version 3.0. Three patients were enrolled at each dose level. If one of three patients experienced a dose-limiting toxicity (DLT) during the first cycle, three additional patients were enrolled at that dose level. If no additional DLTs were observed, then dose escalation proceeded. If two or more patients in a cohort experienced a DLT, then the maximum tolerated dose would be considered exceeded. DLT was defined as treatment-related toxicity occurring within the first 21 days of therapy that included ≥ grade 3 nonhematologic toxicity (excluding fatigue, alopecia, or toxicity attributed to androgen deprivation), hematologic toxicity defined as grade 4 thrombocytopenia or grade 3 thrombocytopenia with bleeding, grade 4 neutropenia persisting for more than 7 days, grade 4 neutropenia associated with fever higher than 38.5C, or removal of a patient from toxicity attributable to treatment. Lymphopenia or anemia of any grade, and toxicities related to androgen deprivation therapy were excluded as DLTs.

Dose modifications were defined according to protocol. Dosages were reduced for day 1 neutrophil count lower than 1,500/mm^3 or platelet count lower than 75,000/mm^3, neutrophil count lower than 500/mm^3 for more than 7 days, neutrophil count lower than 500/mm^3 associated with fever, platelet count lower than 25,000/mm^3, or platelet count lower than 50,000/mm^3 associated with bleeding, and any ≥ grade 3 nonhematologic toxicity related to therapy. Grade 2 or 3 neurotoxicity required ixabepilone dose reduction. Grade 4 and recurrent grade 3 neurotoxicity required ixabepilone discontinuation. Mitoxantrone was discontinued if the ejection fraction decreased below the institutional lower limit of normal and declined by ≥ 15%. For each dose reduction, ixabepilone dose was reduced by 5 mg/m^2, and mitoxantrone dose was reduced by 2 mg/m^2. Patients were removed from treatment if more than two dose reductions were required or if there was a treatment delay of longer than 21 days due to toxicity. Patients were not treated with prophylactic

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antibacterials, and granulocyte growth factor for asymptomatic neutropenia on dose levels I to VI. Secondary prophylaxis with growth factors for recurrent neutropenic infection was allowed in dose levels I to VI.

Statistical Considerations

Successive cohorts of patients were accrued to determine the maximum tolerated dose that resulted in lower than 33% DLTs with the combination of ixabepilone and MP. At least six patients were treated at the maximum dose to increase the likelihood that the risk of a DLT was lower than 33%. Secondary objectives were to obtain initial estimates of response to study therapy based on PSA Working Group 1 criteria and objective responses by RECIST in patients with measurable disease.[8,9]

RESULTS

Patient Characteristics

Between July 2006 and February 2008, 36 patients were enrolled at four participating centers (Table 2). The median age of patients was 66. Seventy-one percent of patients had a Gleason score of 8 to 10. Sixty-four percent of patients had an ECOG performance status of 1 to 2, while 36% had an ECOG performance status of 0. The median lactate dehydrogenase, alkaline phosphatase, and hemoglobin were 313 U/L, 142 U/L, and 11.9 g/dL. The median number of prior chemotherapy cycles was 8.5. Forty-seven percent of patients had experienced a PSA response to prior taxane-based therapy by PSA Working Group 1 criteria. Twenty-three patients (64%) progressed on docetaxel therapy by PSA criteria, nine (25%) progressed on docetaxel therapy in bone and/or soft tissue, two (5.5%) stopped docetaxel therapy for toxicity, and two (5.5%) stopped docetaxel therapy with stable disease after completing a course of chemotherapy. The majority of patients received no intercurrent therapy between docetaxel treatment and enrollment on trial. Two patients received other investigational therapy after docetaxel before enrollment (vorinostat and tandutinib; Millenium Pharmaceuticals, Cambridge, MA), and two other patients received palliative radiotherapy.

Table 2. Patient Characteristics (N = 36)

Characteristic	No.	%
Median age, years	66	
Range	36-79	
Gleason score	35	
6-7	10	
8-10	25	
ECOG PS		
0	13	
1-2	23	
Median PSA, ng/mL	236.93	
Range	12.8-7,167	
Mean No. of prior chemotherapy cycles	8.5	
Range	3-80	
Prior chemotherapy regimens		
Docetaxel	25	
Docetaxel + bevacizumab/placebo	4	
Docetaxel + sunitinib	2	
Docetaxel + estramustine	3	
Docetaxel + valatinib	1	
Docetaxel + diethylstilbestrol	1	
Prior chemotherapy best response		
PSA decline ≥ 50%	17	
Progressive disease	12	
Stable disease	7	
Reason for discontinuing docetaxel		
PSA progression only	23	
Bone progression	3	
Soft tissue progression	5	
Both bone and soft tissue progression	1	
Toxicity	2	
Completed therapy, stable disease	2	
Interval between docetaxel and study therapy, days		
≥ 60	25	
< 60	11	
Baseline laboratory tests		
Median LDH, U/L	313	
Range	118-1,046	
Median alkaline phosphatase, U/L	142	
Range	37-780	
Median hemoglobin, g/dL	11.9	
Range	8.4-14.1	

Abbreviations: ECOG, Eastern Cooperative Oncology Group; PS, performance status; PSA, prostate-specific antigen; LDH, lactate dehydrogenase.

Dose Escalation and DLT

A total of 178 cycles of treatment were administered to 36 patients. No DLTs were observed in the first three cohorts (Table 3). At dose level IV (mitoxantrone 10 mg/m^2, ixabepilone 30 mg/m^2) one patient experienced grade 4 neutropenia lasting longer than 7 days leading to cohort dose expansion. No further DLTs were observed at this dose level. At dose level V (mitoxantrone 12 mg/m^2, ixabepilone 30 mg/m^2), one patient experienced grade 3 diarrhea, leading to cohort expansion. An additional event of grade 4 neutropenia lasting more than 7 days was identified only after dose escalation had occurred to dose level VI. At dose level VI (mitoxantrone 12 mg/m^2, ixabepilone 35 mg/m^2), one patient experienced dose-limiting grade 4 neutropenia lasting longer than 7 days, leading to cohort expansion. In the cohort expansion, a second patient on dose level VI also experienced grade 4 neutropenia lasting longer than 7 days, constituting DLT, and accrual was stopped to this cohort. Based on these toxicities, the study was amended to repeat dose levels V and VI with the addition of pegfilgrastim 6 mg subcutaneously on day 2 (dose levels Va and VIa). No DLTs were observed on dose level Va (mitoxantrone 12 mg/m^2, ixabepilone 30 mg/m^2, pegfilgrastim 6 mg subcutaneous). One patient treated on dose level Va was inadvertently treated with the dose level VIa dose of ixabepilone (5 mg higher than planned) for cycle 1, and was replaced in the dose escalation. As this dose level had been previously tested without pegfilgrastim, the patient was included in the overall toxicity and response reporting. One patient on dose level VIa (mitoxantrone 12 mg/m^2, ixabepilone 35 mg/m^2, pegfilgrastim 6 mg subcutaneous) died of neutropenic infection leading to respiratory and renal failure in the setting of progressive disease during cycle 1 of treatment. That patient was also receiving concomitant therapy with verapamil, a moderate CYP3A4 inhibitor. Dose level VIa was expanded to six patients, and no further DLTs were observed. The maximum tolerated dose with the combination of ixabepilone and mitoxantrone was not exceeded in this study, but further dose escalation was not undertaken, as the study plan was to reach therapeutic dose levels for each drug (mitoxantrone 12 mg/m^2 and ixabepilone 35 mg/m^2) and not escalate further. Furthermore, the treatment-related

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Table 3. DLTs (cycle 1 only) and Responses

Dose Level	DLT Frequency	DLTs	PSA Declines ≥ 50%/ Total Patients	Objective Responses/ Assessable Patients
I	0/3	—	0/3	0/2
II	0/3	—	0/3	0/1
III	0/3	—	1/3	0/2
IV	1/6	Prolonged grade 4 neutropenia	1/6	1/4
V	2/6	Grade 3 diarrhea; prolonged grade 4 neutropenia	2/6	1/5
VI	2/5	Prolonged grade 4 neutropenia × 2	3/5	0/3
Va	0/4		2/4	0/2
VIa	1/6	Grade 5 neutropenic infection	2/6	0/1

Abbreviation: DLT, dose-limiting toxicities.

death on the highest dose level suggested that further dose escalation was not warranted. Based on the observed DLTs, the recommended phase II dose is mitoxantrone 12 mg/m^2 and ixabepilone 35 mg/m^2 day 1, pegfilgrastim 6 mg on day 2, and prednisone 5 mg twice daily continuously.

Overall Toxicity

As anticipated, hematologic toxicity was frequently observed (Table 4). Grade 3 neutropenia was observed in 28% of patients, and grade 4 neutropenia was observed in 31% of patients. Grade 4 neutropenia lasting longer than 7 days was observed in 11% of patients. Grade 3/4 neutropenia was observed in 33% of all treatment cycles. Grade 3 thrombocytopenia and grade 3 anemia were infrequent (6% and 8% respectively), and no patients experienced grade 4 thrombocytopenia or grade 3 thrombocytopenia with bleeding.

Nonhematologic toxicity related to study therapy is detailed in Table 5. Cardiovascular toxicity included two patients with grade 2 asymptomatic decreased left ventricular ejection fraction (to 40% to 50%), and two patients with atrial fibrillation (one grade 2, another grade 3). Grade 3 motor neuropathy was observed in one patient, grade 2 motor neuropathy was observed in one patient, and grade 2 sensory neuropathy was observed in one patient.

Response Evaluation

Anticancer activity was assessed as a secondary end point of this study. Partial objective RECIST-defined responses were observed in two of 20 patients with measurable disease: one on dose level IV and one on dose level V. In addition, 11 patients (31%) experienced confirmed PSA declines ≥ 50% (Table 3 and online-only Appendix Fig A1). Of the 21 patients who received the US Food and Drug Administration–approved mitoxantrone dose of 12 mg/m^2 (dose levels V, Va, VI, and VIa), nine patients (43%) experienced confirmed PSA declines ≥ 50% (Fig 1; 95% CI, 22% to 66%). For responders, the median time to progression was 5.3 months (range, 3.0 to 11.1).

Table 4. Frequency of Grade 3 and 4 Hematologic Toxicity Across All Dose Levels and All Cycles

	Grade 3		Grade 4	
Toxicity	No.	%	No.	%
Neutropenia	10	28	11	31
> 7 days	9	25	4	11
Febrile neutropenia	1	3	—	—
Lymphopenia	12	33	2	6
Leukopenia	10	28	6	17
Thrombocytopenia	2	6	—	—
Anemia	3	8	—	—

DISCUSSION

While docetaxel chemotherapy is associated with an overall survival benefit for patients with castration-resistant prostate cancer, the median time to progression remains short, and overall survival remains fewer than 2 years. Recently reported data demonstrated that satraplatin did not provide a survival benefit when compared with prednisone alone in CRPC patients previously treated with chemotherapy.[10] Thus, the exploration of new therapeutic approaches for these patients is clearly warranted.

In a previously reported randomized phase II trial, ixabepilone and MP appeared to have clinical noncrossresistance as second- and third-line therapy for CRPC.[7] Several patients who progressed on one arm and crossed over to the other therapy demonstrated responses. Based on these data, the current study evaluated the safety and tolerability of the combination of ixabepilone and MP as second-line chemotherapy in patients with metastatic CRPC who had progressed during or after a single taxane-based chemotherapy regimen.

This study has demonstrated that mitoxantrone and ixabepilone can generally be safely administered in combination at doses that have demonstrated single-agent activity in CRPC. The recommended phase II dose is mitoxantrone 12 mg/m^2 and ixabepilone 35 mg/m^2 administered intravenously every 21 days, along with prednisone 5 mg orally twice per day continuously. Treatment was well tolerated in most patients. However, treatment at these dose levels required pegfilgrastim to prevent prolonged neutropenia. High rates of neutropenia have been observed with mitoxantrone-based chemotherapy in prostate cancer. For example, grade 3/4 neutropenia was observed in 59% of patients treated with mitoxantrone, without a concomitant high incidence of neutropenic infections or morbidity.[5] The low frequency of febrile neutropenia may be explained by the relatively low frequency of severe mucositis observed with mitoxantrone. In this phase I study, no patients experienced grade 3 or 4 mucositis.

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Table 5. Grade 2 and Higher Treatment-Related Maximal Nonhematologic Toxicity, All Dose Levels, and All Cycles

	Grade					
	2		3		4	
Toxicity	No.	%	No.	%	No.	%
Alopecia	1	3	—	—	—	—
Anorexia	2	6	—	—	—	—
ARDS	1	3	—	—	—	—
Atrial fibrillation	1	3	1	3	—	—
Bone pain	2	6	—	—	—	—
Chest pain	1	3	—	—	—	—
Dehydration	—	—	1	3	—	—
Diarrhea	1	3	1	3	—	—
Dizziness	1	3	—	—	—	—
Dyspepsia	1	3	—	—	—	—
Dyspnea	1	3	—	—	—	—
Edema limbs	1	3	—	—	—	—
Fatigue	11	31	—	—	—	—
Febrile neutropenia	1	3	—	—	—	—
Fever	1	3	—	—	—	—
Hot flashes	1	3	—	—	—	—
Infection	1	3	—	—	2	6
Muscle weakness	1	3	1	3	—	—
Nausea	3	8	—	—	—	—
Pain	1	3	—	—	—	—
Peripheral motor neuropathy	1	3	1	3	—	—
Peripheral sensory neuropathy	1	3	—	—	—	—
Phlebitis	1	4	—	—	—	—
Pleural effusion	1	4	—	—	—	—
Pneumonia	1	3	2	6	—	—
Reduced LVEF	2	6	—	—	—	—
Renal failure	—	—	—	—	1	3
Syncope	—	—	1	3	—	—
Taste alteration	2	6	—	—	—	—
Vomiting	2	6	1	3	—	—
Weight loss	2	6	—	—	—	—

Abbreviations: ARDS, adult respiratory distress syndrome; LVEF, left ventricular ejection fraction.

While further dose escalation was formally possible beyond doses of mitoxantrone 12 mg/m^2 and ixabepilone 35 mg/m^2, due to concerns of broad applicability of the regimen to the general population of CRPC patients, further dose escalation was not pursued.

Neurotoxicity was not frequently observed in this study despite the sequential use of two potentially neurotoxic agents (docetaxel and ixabepilone). Only 12% of patients experienced grade 2 or greater neuropathy. These data are consistent with the previous study of ixabepilone monotherapy after taxanes in CRPC.[7] Patients with preexisting grade 2 or higher neuropathy were excluded from participation in this trial. These data are also similar to what has been observed in taxane-refractory metastatic breast cancer patients treated with ixabepilone.[11,12] As a result, it may be that these criteria selected for a patient population less susceptible to neuropathy. Furthermore, variations in assessment of toxicity between different physicians and different institutions may result in under-reporting of grade 3 neuropathy.

Although efficacy was not a primary end point of this study, the frequency of PSA declines observed with the combination is intriguing. In patients who received treatment on this study with the US Food and Drug Administration–approved doses of mitoxantrone, the PSA response frequency was 42%, while the PSA response rate for salvage mitoxantrone and prednisone has been reported to be 20%.[7] The PSA response frequency in first-line mitoxantrone studies ranges from 19% to 32%.[1,2,5] While the numbers of patients in this study are small, these results indicate that the addition of ixabepilone to MP may result in at least additive efficacy. While PSA declines are an intermediate end point and not a direct measure of clinical benefit, the PSA Working Group Consensus Criteria were developed precisely to screen for the activity of cytotoxic regimens in men with metastatic CRPC. The preliminary activity demonstrated in this study suggests that this regimen is worthy of further evaluation. The DOD Prostate Cancer Clinical Trials Consortium is testing this regimen in ongoing phase II study.

One potential weakness of this study may result from patient selection resulting in a group of patients not accurately reflecting the distribution of CRPC patients in the community. In fact, in an earlier study of mitoxantrone versus ixabepilone, such selection was mandated by virtue of an eligibility requirement of progression while on docetaxel or within 60 days of the last docetaxel dose. This study had no such restrictions, and, in fact, 31% of patients accrued to this study developed progressive disease more than 60 days after

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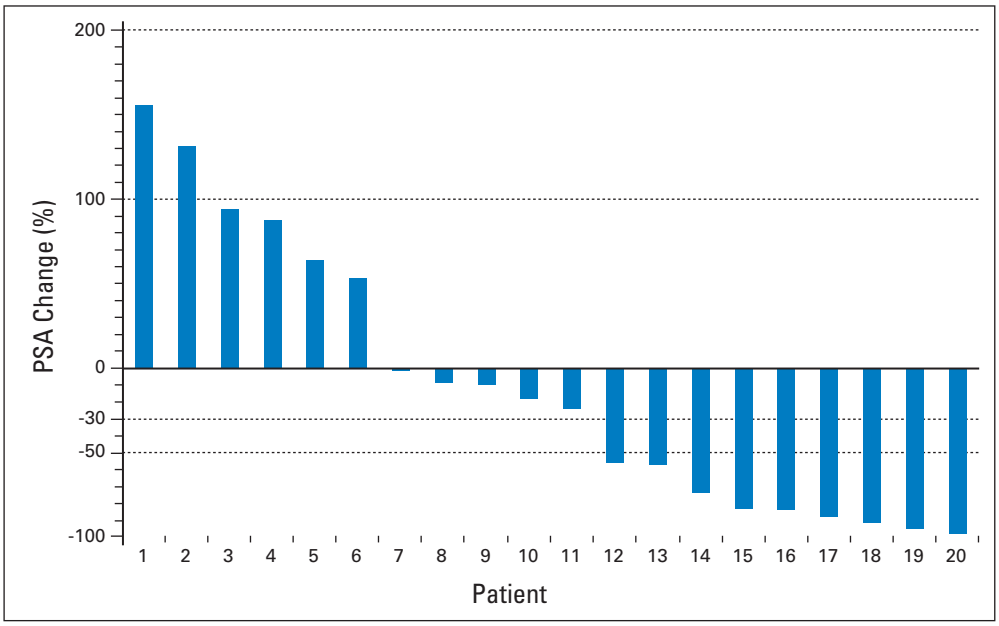

Fig 1. Prostate-specific antigen (PSA) waterfall plot of maximum PSA declines of the 21 patients who received the US Food and Drug Administration–approved mitoxantrone dose of 12 mg/m^2 (dose levels V, Va, VI, and VIa). Nine patients (43%) experienced confirmed PSA declines of at least 50% (95% CI, 22% to 66%).

the last docetaxel dose. It is possible that some of these patients might have responded to rechallenge with docetaxel. While this difference may result in inadvertent selection of "better" patients for this study, it also reflects the broad distribution of taxane pretreated patients in the community.

A second potential weakness involving careful patient selection at a single specialized center is somewhat addressed by the multicenter participation in this trial. Nevertheless, this study, conducted in four high volume prostate cancer tertiary referral centers, demonstrates that combination chemotherapy for second-line chemotherapy for CRPC is feasible with these agents.

In summary, the combination of ixabepilone and mitoxantrone with pegfilgrastim is safe and feasible in metastatic CRPC patients who have developed progressive disease despite docetaxel-based therapy. Furthermore, this regimen has demonstrated sufficient activity to warrant phase II testing.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Original Article

Ixabepilone, Mitoxantrone, and Prednisone for Metastatic Castration-Resistant Prostate Cancer After Docetaxel-Based Therapy

A Phase 2 Study of the Department of Defense Prostate Cancer Clinical Trials Consortium

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BACKGROUND: Mitoxantrone plus prednisone and ixabepilone each have modest activity as monotherapy for second-line chemotherapy in patients with docetaxel-refractory castration-resistant prostate cancer. Clinical noncross-resistance was previously observed. Phase 1 testing determined the maximum tolerated dose and dose-limiting toxicities with the combination regimen; a phase 2 study was conducted to evaluate the activity of the combination. **METHODS:** Patients with metastatic progressive castration-resistant prostate cancer during or after 3 or more cycles of taxane-based chemotherapy enrolled in a phase 2 multicenter study of ixabepilone 35 mg/m^2 and mitoxantrone 12 mg/m^2 administered on Day 1 every 21 days with pegfilgrastim support, along with prednisone 5 mg twice daily. Patients were evaluated for disease response and toxicity. **RESULTS:** Results are reported for the 56 evaluable patients. Twenty-five (45%; 95% confidence interval [CI], 31%-59%) experienced confirmed $\geq$50% prostate-specific antigen (PSA) declines, 33 (59%; 95% CI, 45%-72%) experienced confirmed $\geq$30% PSA declines, and 8 of 36 patients (22%; 95% CI, 10%-39%) with measurable disease experienced objective responses. Median time to PSA or objective progression was 4.4 months (95% CI, 3.5-5.6), and median progression-free survival was also 4.4 months (95% CI, 3.0-6.0). Median overall survival was 12.5 months (95% CI, 10.2-15.9). Thirty-two percent of patients experienced grade 3 of 4 neutropenia, and 11% experienced grade 3 or higher neutropenic infections, including 1 treatment-related death. Grade 2 and 3 neuropathy occurred in 11% and 12.5% of patients, respectively. **CONCLUSIONS:** These results suggest that the combination of ixabepilone and mitoxantrone is both feasible and active in castration-resistant prostate cancer and requires dosing with pegfilgrastim. ***Cancer* 2011;00:000–000.** *© 2010 American Cancer Society.*

KEYWORDS: prostate cancer, chemotherapy, metastatic, mitoxantrone, ixabepilone, docetaxel.

Mortality in prostate cancer is primarily related to the development of metastatic castration-resistant disease, and options after docetaxel, the first-line standard of care, remain limited.[1] Recent data have established cabazitaxel as the standard second-line therapy.[2] Mitoxantrone with prednisone, which has been demonstrated to improve quality of life as front-line therapy, has been used extensively, with 50% PSA declines reported in 20% of patients previously treated with docetaxel.[3-5] Ixabepilone, an epothilone analog, has similarly been demonstrated to have a 17% response rate in this setting. Of interest, objective responses to mitoxantrone/prednisone after second-line ixabepilone and conversely to ixabepilone after second-line mitoxantrone/prednisone were observed during a randomized phase 2 study, suggesting there is noncross-resistance with the 2 regimens.

On the basis of the nonoverlapping toxicity of these regimens and their apparent noncross-resistance, a phase 1 study combining these agents was undertaken in patients previously treated with docetaxel.[6] The combination was well

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tolerated. Although hematologic toxicity required treatment with pegfilgrastim, other toxicity, including neurotoxicity, was modest. The regimen recommended for phase 2 testing was mitoxantrone 12 mg/m^2 and ixabepilone 35 mg/m^2, given with prednisone 5 mg twice daily, along with pegfilgrastim 6 mg on Day 2. Responses, as defined by a $\geq$50% PSA decline, were observed in 31% of patients, with objective responses in 2 of 36 patients in the phase 1 study. When limited to the 21 patients treated with 12 mg/m^2 of mitoxantrone plus ixabepilone at a dose of 30 mg/m^2 or higher, 43% of patients experienced prostate-specific antigen (PSA) declines of $\geq$50% (95% confidence interval [CI], 22% to 66%). When compared with the response proportions reported for monotherapy with either ixabepilone or mitoxantrone of approximately 20%, these results suggested at least additive effects of the 2 agents and were sufficiently promising to warrant a phase 2 study to determine the activity of this novel regimen.

MATERIALS AND METHODS

Study Design

This study was a multicenter, single-arm, phase 2 study of ixabepilone and mitoxantrone with prednisone in castration-resistant prostate cancer patients who developed progressive disease during or after docetaxel-based chemotherapy. This study was undertaken in the Department of Defense Prostate Cancer Clinical Trials Consortium, with accrual occurring at 6 academic centers. The primary endpoint of the study was the proportion of patients achieving $\geq$50% PSA declines. Secondary endpoints included overall safety, the frequency of objective responses, time to progression, progression-free survival, and overall survival. This study was approved by the Clinical Trial Evaluation Program of the National Cancer Institute, the Prostate Cancer Clinical Trials Consortium Review Committee, and the local institutional review boards of participating institutions. All patients provided written informed consent.

Eligibility

Patients were required to have histologically confirmed prostate cancer with metastatic spread and progressive disease despite castrate testosterone levels. Patients were required to have received at least 3 cycles of taxane-based chemotherapy, and only 1 prior chemotherapy regimen was permitted. For patients with measurable disease, progression was defined according to Response Evaluation Criteria in Solid Tumors (RECIST), and for patients without measurable disease, a PSA of $\geq$2 ng/mL and a bone scan consistent with metastasis were required. Patients without measurable disease were required to have either PSA progression or a bone scan demonstrating 1 or more new metastatic lesions. PSA progression was defined according to PSA Working Group 1 criteria.[7] Patients were required to have an Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 2 and $\leq$grade 1 peripheral neuropathy (National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0). Patients who had not undergone prior orchiectomy were required to remain on a luteinizing hormone-releasing hormone agonist. Other hormonal therapy, with the exception of prednisone 5 mg twice daily, as given with docetaxel, was not allowed within 4 weeks of study entry. Docetaxel was not allowed within 4 weeks of enrollment. No prior mitoxantrone or ixabepilone was allowed. Radiation or radiopharmaceutical therapy must have been completed at least 4 and 8 weeks, respectively, before enrollment. Cardiac ejection fraction was required to be above the lower limit of normal for the institution. Patients with clinically significant cardiovascular disease, including New York Heart Association class III or IV heart failure, active angina, or a history of myocardial infarction within 6 months, were excluded. Laboratory requirements included testosterone $<$50 ng/dL; creatinine $\leq 1.5 \times$ upper limit of normal (ULN) or calculated creatinine clearance $\geq$40 mL/min; alanine aminotransferase (ALT) and aspartate aminotransferase (AST) $<2.5 \times$ ULN; granulocytes $\geq$2000/mm^3; platelets $\geq$100,000/mm^3; and total bilirubin $\leq 1.5 \times$ ULN. Because ixabepilone is a CYP3A4 substrate, concurrent use of moderate to strong CYP3A4 inhibitors was prohibited.

Study Therapy

Patients were treated on day 1 of 21-day cycles. Premedication with oral H1- and H2-blockers was administered 1 hour before treatment to prevent hypersensitivity reactions. Patients received mitoxantrone 12 mg/m^2 intravenously over 30 minutes. Ixabepilone 35 mg/m^2 was subsequently administered as a continuous infusion over 3 hours. Patients were monitored for hypersensitivity reactions for 1 hour. If grade 2 to 4 hypersensitivity reactions developed despite antihistamine premedication, corticosteroid premedication was used for subsequent cycles. Prednisone was administered 5 mg twice daily continuously. Pegfilgrastim 6 mg was administered subcutaneously on Day 2. Patients were treated until disease

progression, unacceptable toxicity, or patient preference to discontinue therapy.

Assessment for Response and Toxicity

Patients were assessed with chest x-ray or chest computed tomography (CT), CT of the abdomen and pelvis, and bone scan every 3 cycles. PSA, complete blood count with differential and platelets, electrolytes, blood urea nitrogen, creatinine, magnesium, lactate dehydrogenase, albumin, AST, ALT, total bilirubin, and alkaline phosphatase were obtained every cycle. Physical examination and assessment of performance status were undertaken each cycle. Echocardiogram or MUGA (Multi Gated Acquisition) Scan was performed at baseline, every 3 cycles, and as clinically indicated.

Objective response was defined by RECIST, and both 50% and 30% PSA declines were determined, with a repeat PSA required 3 weeks later for confirmation.[7,8] Disease progression was defined as new metastases outside of the bone, $\geq$1 new bone lesions confirmed on repeat imaging, a need for radiation while on therapy, unequivocal progression of nontarget lesions, progression by RECIST, or PSA progression. PSA progression was defined according to PSA Working Group 1 criteria, with a PSA increase of 25% above the nadir value, occurring at least 9 weeks (3 cycles) after initiating the study.

Toxicity was monitored by history, physical examination, and laboratory assessment before each cycle. Adverse events were graded according to National Cancer Institute Common Toxicity Criteria version 3.0. For grade 3 or higher toxicities, both ixabepilone and mitoxantrone were held until resolution to $\leq$grade 1, then reinstituted at 5 mg/m^2 less of ixabepilone and 2 mg/m^2 less of mitoxantrone. The same process was required for recurrent toxicities, with a third recurrence resulting in removal from study therapy. For corticosteroid toxicity, prednisone doses could be modified without removing a patient from protocol therapy. For neurotoxicity secondary to ixabepilone, therapy was held for grade 2 or 3 toxicity but otherwise managed as above. Alopecia, lymphopenia, anemia, and toxicities related to androgen deprivation were excluded as dose-limiting or modifying criteria.

Statistical Considerations

The primary endpoint of this study was the proportion of patients responding to treatment defined as observing a PSA decline of $\geq$50% (PSA response) based on PSA Working Group 1 criteria. Treatment of 58 patients allowed for the detection of a PSA response proportion of 35%, compared with a null hypothesis of 20% with a power of 0.90 and a level of significance of 0.10. Simon's MiniMax 2-stage design was used for accrual, to allow for an interim analysis for efficacy after the first 33 patients had been accrued and had been followed for 3 cycles of treatment. Had 6 or fewer of the first 33 patients enrolled demonstrated a PSA decline of $\geq$50%, accrual would have been terminated, resulting in a probability of early termination if the null hypothesis were true of 50%. Objective responses were evaluated according to RECIST for patients with measurable disease. Descriptive statistics were calculated to characterize the patient cohort, baseline disease parameters, outcome, and toxicity. The time to progression, progression-free survival, and overall survival were measured from the start of protocol therapy and evaluated using the Kaplan-Meier product limit method.

RESULTS

Patient Characteristics

Between November 2007 and March 2009, 58 patients were enrolled at 6 member institutions of the Department of Defense Prostate Cancer Clinical Trials Consortium. Two patients were ineligible: 1 because of pre-existing spinal cord compression and 1 because of a secondary diagnosis of colon cancer diagnosed after 2 cycles of therapy; therefore, 56 evaluable patients were included in these analyses. Four patients did not complete the minimum 3 cycles of therapy defined by the protocol to be necessary for response assessment; 2 discontinued for progressive disease and 2 withdrew because of concerns over rising PSA. These 4 patients are included in both efficacy and toxicity analyses. Patient characteristics are summarized in Table 1. The median age of patients at the start of protocol therapy was 66.7 years. Sixty-nine percent of patients had a Gleason score of 8 to 10. Sixty-six percent had an ECOG performance status of 1 to 2, and 34% had an ECOG performance status of 0. The median PSA was 171.2 (range, 2.79-3717.1), and the median alkaline phosphatase was 134 (range, 42-1094). All patients had received prior docetaxel therapy once every 3 weeks. The median number of prior chemotherapy cycles was 8 (range, 3-33). The median prior treatment duration was 6.4 months (range, 2.2-29.1), and the median time between discontinuation of docetaxel and initiation of study therapy was 53 days (range, 5-413). Fifty percent of patients (28 of 56) had experienced a PSA response to prior taxane-based therapy by PSA Working Group 1

Table 1. Patient Characteristics (N=56)

Median age at entry (range)	66.7 (47-83)
ECOG PS at protocol entry, patients (%)	
0	19 (34)
1-2	37 (66)
Gleason score at diagnosis (n=54), patients (%)	
4-6	3 (5.5)
7	14 (26)
8-10	37 (68.5)
Median PSA, ng/mL (range)	171.2 (2.79-3717.1)
Baseline laboratory results at protocol entry	
Median LDH, IU/L (range)	290 (123-2333)
Median alkaline phosphatase, U/L (range)	134 (42-1094)
Median hemoglobin, g/dL (range)	11.7 (9.3-14.1)
Prior chemotherapy: best response, patients (%)	
PSA response/partial response	28 (50)
Stable disease for patients with objective disease	18 (32)
Progressive disease	10 (18)
Prior 3-week chemotherapy cycles, median No. (range)	8 (3-33)
Median duration, mo (range)	6.4 (2.2-29.1)
Median duration from end of taxane, d (range)	53 (5-413)
Study treatment	
Cycles received, median No. (range)	5+ (1-13)
Still on treatment, patients	1[a]

ECOG indicates Eastern Cooperative Oncology Group; PS, performance status; PSA, prostate-specific antigen; LDH, lactate dehydrogenase.
[a] Duration 10.4 months.

Table 2. Response Data

Response	No.	%
≥30% PSA decline	33	59
≥30% PSA decline by 12 weeks	31	55
≥50% PSA decline	25	45
≥50% PSA decline by 12 weeks	17	30
Objective responses	8/36	22

PSA indicates prostate-specific antigen.

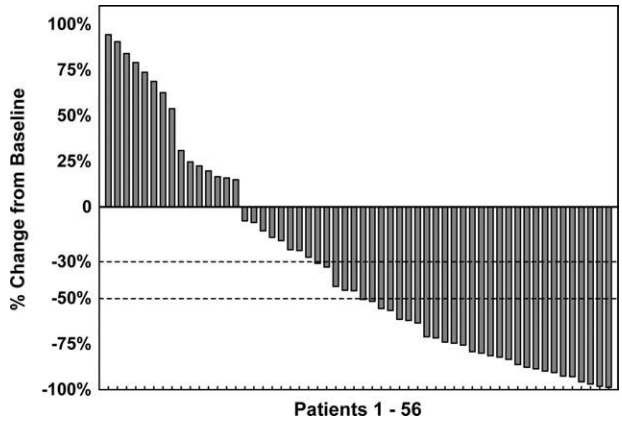

Figure 1. Maximum percentage change in prostate-specific antigen is shown.

criteria, whereas half of the enrolled patients never had a PSA response to docetaxel therapy. Fifty-nine percent of patients had subsequently progressed on docetaxel therapy by PSA criteria alone, 30% had radiographic progression, 9% stopped docetaxel therapy for toxicity, and 2% stopped with stable disease after completing a planned course of therapy. Thus, 89% of patients had developed docetaxel-resistant castration-resistant prostate cancer before enrolling on this trial. Twenty-five percent (14 patients) of patients received therapy after docetaxel but before beginning this study, including ketoconazole (n = 5), sunitinib (n = 3), bicalutamide (n = 2), palliative radiotherapy (N = 2), PSMA ADT (an antibody against prostate specific membrane antigen), and GVAX (a vaccine consisting of prostate cancer cells modified to secrete granulocyte-macrophage colony-stimulating factor), 1 each.

Clinical efficacy to ixabepilone and mitoxantrone with prednisone chemotherapy is reported for all 56 eligible patients (Table 2). Overall, 25 (45%) patients experienced confirmed PSA declines of ≥50% (Fig. 1; 95% CI, 31%-59%), and 33 (59%) had confirmed PSA declines of ≥30% (95% CI, 45%-72%). After 12 weeks of protocol therapy, 30% of the patients achieved PSA declines of at least 50%, indicating that the study null hypothesis of 20% can be rejected (1-sided binomial exact test: $P = .04$). Partial objective RECIST-defined responses were observed in 8 patients of 36 with measurable disease (22%; 95% CI, 10%-39%).

With a median follow-up of 9.9 months (range, 3.1-19.4) from the start of protocol therapy, the median time to progression was 4.4 months (95% CI, 3.5-5.6). The median PSA or objective progression-free survival was also 4.4 months (Fig. 2; 95% CI, 3.0-6.0), and the median overall survival was 12.5 months (Fig. 3; 95% CI, 10.2-15.9).

Patients with a prior response to docetaxel therapy were as likely to respond to ixabepilone and mitoxantrone with prednisone second-line therapy as patients with no prior response to docetaxel. Of the 28 patients who had a ≥50% PSA decline with docetaxel-based therapy, 39% had a ≥50% PSA decline with ixabepilone and mitoxantrone with prednisone. Of the 10 patients whose best

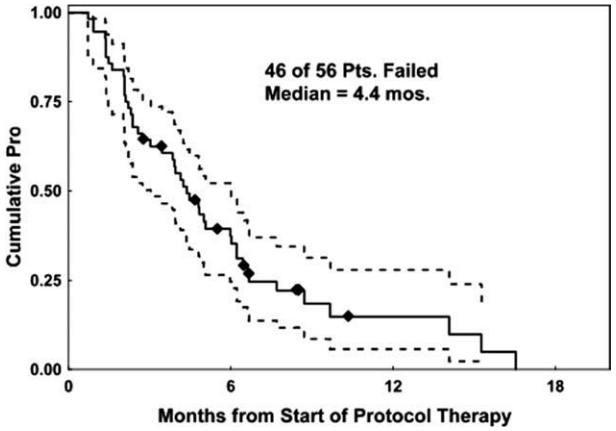

Figure 2. Progression-free survival with ixabepilone and mitoxantrone with prednisone is shown. Pro indicates progression; Pts., patients.

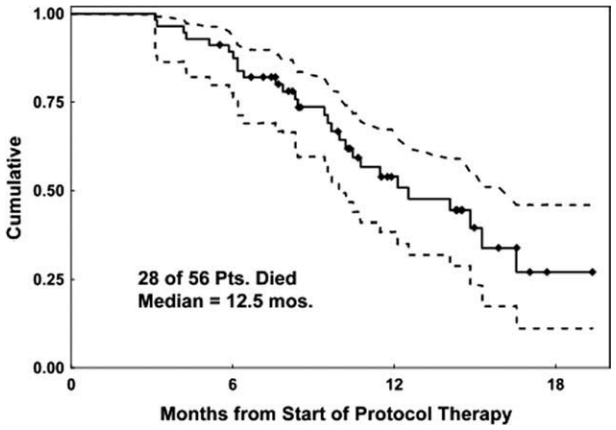

Figure 3. Overall survival with ixabepilone and mitoxantrone with prednisone is shown.

response to docetaxel-based therapy was progressive disease, 40% had a $\geq$50% PSA response to ixabepilone and mitoxantrone with prednisone ($P = .71$).

Toxicity

Toxicity data are reported for all 56 eligible patients and are summarized in Table 3. Thirty-two percent of patients experienced grade 3 or 4 neutropenia. Eleven percent of patients had neutropenia associated with infection. Five grade 3 infections occurred in 5 patients (2 pulmonary, 1 skin, 1 *Clostridium difficile* colitis, 1 septic arthritis of the elbow), and 1 grade 4 bacteremia occurred. One treatment-associated death occurred in the 1 patient on study on verapamil, a moderate CYP3A4 inhibitor. This patient

Table 3. Toxicity Related to Study Therapy

Adverse Event	Grade 3	Grade 4	Grade 5
Hematologic			
Leukopenia	9	11	
Lymphopenia	17	3	
Neutropenia	6	10	
Anemia	3	1	
Thrombocytopenia	7	3	
Nonhematologic			
Allergic reaction	1		
AST/ALT increased	1		
Dyspnea	2		
Fatigue	5		
Hyperbilirubinemia	1		
Hypoalbuminemia	1		
Infection	5[a]	1[a]	1[b]
Hypocalcemia	1		
Hypophosphatemia	1		
Mucositis	1		
Nausea/vomiting	1		
Neuropathy	7		
Vasovagal episode		1	

AST indicates aspartate aminotransferase; ALT, alanine aminotransferase.
[a] Sites of infection: skin (cellulitis), blood (methicillin-resistant *Staphylococcus aureus*, grade 4), pneumonia (2), colon (*Clostridium difficile* colitis), elbow (septic arthritis). All but septic arthritis associated with neutropenia. The *C. difficile* infection occurred in a patient with pneumonia treated with antibiotics.
[b] There was 1 treatment-related death in a patient with urosepsis and neutropenia who was on verapamil.

experienced urosepsis in association with neutropenia. Grade 3 or higher thrombocytopenia and anemia were uncommon (18% and 7%, respectively). Cardiovascular toxicity included 1 grade 4 cardiac infarct, 1 grade 3 atrial fibrillation, and 1 grade 2 decrease in ejection fraction. Grade 2 and 3 sensory neuropathy was observed in 6 and 7 patients (11% and 13%), respectively. Other toxicities of note included grade 2 fatigue in 13 patients and grade 3 fatigue in 5 patients.

Treatment Administered

Patients were removed from study therapy primarily for progressive disease. Twenty-seven and 9 patients (48% and 16%) discontinued protocol treatment because of PSA and objective progression, respectively, and 4 (7%) others had both PSA and objective disease progression. Ten (18%) patients discontinued therapy for toxicity after a median of 7 cycles (range, 1-13). Two (4%) patients discontinued after completing 12 cycles, and 3 (5%) patients withdrew, 2 because of concerns over rising PSA, and 1 because of a combination of toxicity and concerns over rising PSA. One (2%) patient remains on therapy 10.6 months from the start of protocol therapy having received 8 cycles of therapy to date.

DISCUSSION

After progression on docetaxel-based chemotherapy, chemotherapy options for patients with metastatic castration-resistant prostate cancer remain poor. Recently reported data suggest that cabazitaxel may represent an important therapeutic option for patients with progressive disease after docetaxel.[2] Mitoxantrone with prednisone is often used as second-line therapy but is associated with a PSA response rate of only 20%.[5] Ixabepilone also has a disappointing PSA response rate of 17% after docetaxel. The objective response rates associated with ixabepilone monotherapy and mitoxantrone with prednisone after docetaxel are also low at 4% and 10%, respectively. On the basis of results from a randomized phase 2 study suggesting that ixabepilone and mitoxantrone with prednisone have noncross-resistance and a phase 1 trial of the ixabepilone and mitoxantrone with prednisone combination demonstrating surprisingly high activity, the present phase 2 trial was undertaken.[5]

The ixabepilone and mitoxantrone with prednisone regimen was found to have significant activity, with a PSA response proportion of 45%, and an equally promising objective response proportion of 22%. The overall survival in this group of patients was 12.5 months. Although direct comparisons are not possible across studies, and differences in patient populations may account for results observed, it is notable that the overall survival was 10.4 months on the ixabepilone arm (with mitoxantrone on progression) and 9.8 months on the mitoxantrone arm (with ixabepilone on progression) in the randomized phase 2 study of ixabepilone or mitoxantrone after docetaxel. The time to progression of 4.4 months also appears favorable in comparison to the 2.3-month time to progression on mitoxantrone monotherapy in the randomized phase 2 study.

Data from a randomized phase 3 study comparing cabazitaxel to mitoxantrone with prednisone in patients who had progressed after docetaxel-based therapy indicated that cabazitaxel was associated with a PSA response proportion of 39%, in comparison to 18% on the mitoxantrone/prednisone arm. Although these results cannot be directly compared with the results of the current study of ixabepilone with mitoxantrone and prednisone, the response proportion of 45% in the current study suggests further study may be warranted.

Of interest, response to ixabepilone and mitoxantrone with prednisone does not appear to be dependent on prior response to docetaxel. Although definitive conclusions cannot be drawn given the small numbers of patients, these data suggest that there is no significant cross-resistance between docetaxel and ixabepilone/mitoxantrone with prednisone, and that ixabepilone and mitoxantrone with prednisone therapy may be useful in patients with progressive disease after docetaxel, regardless of docetaxel sensitivity.

The combination of these 2 agents did not appear to result in a dramatic increase in toxicity. Although comparison across studies is fraught with difficulty, toxicity with the study regimen appears to be similar to that associated with mitoxantrone/prednisone use in the second-line alone. In the randomized phase 2 study of mitoxantrone/prednisone and ixabepilone monotherapy, 10% of the 41 patients on the mitoxantrone/prednisone second-line arm experienced febrile neutropenia, and 9% of the 56 patients on this study of the combination (with pegfilgrastim support) experienced febrile neutropenia. It is important to note, however, that this margin of safety can be achieved with the ixabepilone and mitoxantrone with prednisone regimen at the doses studied only with pegfilgrastim support.

Sixteen percent of patients discontinued therapy for toxicity in this phase 2 study of the combination, a number that appears to be similar to the number of patients discontinuing docetaxel as first-line treatment for toxicity. In the randomized phase 2 study of mitoxantrone or ixabepilone, 10% of the 41 patients on mitoxantrone discontinued therapy for toxicity.[1]

Nonhematologic toxicity was minimal. Despite substantial doses of mitoxantrone (66% of patients received >6 cycles), minimal cardiac toxicity was observed. Similarly, less neuropathy was observed than expected in this taxane-pretreated population, with 11% and 12.5% of patients developing grade 2 and 3 neurotoxicity, respectively. However, these results may reflect patient selection. As with the prior second-line ixabepilone prostate cancer studies, patients with grade 2 or higher neuropathy at baseline after docetaxel were excluded. This may have selected a patient population less likely to experience neuropathy. Nevertheless, neuropathy was comparable to that seen in breast cancer studies[9-13] in which 12% to 20% of patients develop grade 3 neurotoxicity.

One potential weakness of this study is that the eligibility criteria did not require a previous history of progression while receiving docetaxel-based therapy, but rather required disease progression during or after docetaxel therapy, possibly selecting for a more chemotherapy-sensitive population. However, 89% of the patients on study had, in fact, progressed while receiving docetaxel therapy, suggesting that this study enrolled patients with

docetaxel resistance. Furthermore, there did not appear to be a difference in response proportion as a function of prior response to docetaxel, although small numbers limit this analysis.

Another potential criticism of this study is that the primary endpoint, the proportion of patients achieving a $\geq$50% decline in PSA, per PSA Working Group Criteria, is of uncertain clinical significance. However, the PSA Working Group criteria were initially established to be used specifically in this setting, as a screen for the activity of cytotoxic agents in the phase 2 setting.[7] In addition, the objective response proportion, time to progression, and overall survival observed with ixabepilone and mitoxantrone with prednisone therapy all appeared to be favorable compared with that associated with mitoxantrone monotherapy, suggesting that the high proportion of patients with an observed PSA decline may be associated with improved survival outcomes. Definitive evidence of benefit can only be established by evaluating overall survival in a phase 3 study.

In summary, the combination of ixabepilone and mitoxantrone with prednisone appears to have greater activity than either mitoxantrone or ixabepilone alone in the second-line setting for castration-resistant prostate cancer, and suggests at least additive if not synergistic activity in a disease state where improvement in outcome is needed and long overdue. The combination is well tolerated, although some hematologic toxicity is present and dosing with pegfilgrastim is required. The results of this study suggest that it is appropriate to study further the ixabepilone and mitoxantrone with prednisone regimen in patients with docetaxel-resistant castration-resistant prostate cancer.

CONFLICT OF INTEREST DISCLOSURES

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Functional phenotyping and genotyping of circulating tumor cells from patients with castration resistant prostate cancer

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ABSTRACT

Circulating tumor cells (CTCs) hold promise for studying advanced prostate cancer. A functional collagen adhesion matrix (CAM) assay was used to enrich CTCs from prostate cancer patients' blood. CAM ingestion and epithelial immuno-staining identified CTCs, which were genotyped using oligonucleotide array comparative genomic hybridization. The highest CTC counts were observed in men with metastatic castration resistant prostate cancer (CRPC) compared to castration sensitive prostate cancer. Copy number profiles for CRPC CTCs were similar to paired solid tumor DNA, and distinct from corresponding DNA from the residual CAM-depleted blood. CAM CTC enrichment may allow cellular and genetic analyses in prostate cancer.

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1. Introduction

Prostate cancer is the second leading cause of male cancer related deaths in the United States, accounting for approximately 30,000 deaths annually [1]. Ultimately, resistance to androgen deprivation therapy and chemotherapy are the underlying causes of mortality in patients with advanced prostate cancer and the mechanisms of this resistance are still not understood. A major constraint in studying resistance mechanisms in advanced prostate cancer has been the limited accessibility to metastatic tissue, because biopsy of metastatic prostate cancer is often difficult and impractical, and the yield of cancer cells is low.

In most primary epithelial tumors, a rare subset of malignant epithelial cells emigrates to the bloodstream, and proliferates at distant sites [2,3]. Analysis of circulating tumor cells potentially allows for increased understanding of how cancer metastasizes with subsequent clinical application of this knowledge [4,5]. The majority of CTC detection methods available to date are based on enrichment of the tumor cells from blood using antibodies against epithelial surface antigens, as no cancer-specific markers exist yet [4,5]. Current methods including immuno-affinity and density gradient centrifugation enrichment procedures isolate cell populations that require further characterization of viability and metastatic propensity because the majority of tumor cells in the circulation are dead or dying [3,6,7]. Furthermore, current antibody-based CTC detection in prostate cancer has thus far only demonstrated clinical utility as a prognostic tool for classifying patients into high- and low-risk categories [8–11]. An alternative cell enrichment approach of isolating viable tumor cells from blood is required for biomarker discovery and development for more effective future clinical applications.

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The proclivity of a tumor cell to invade collagenous matrices is one of the hallmarks of metastasis [12,13]. We reasoned that an enrichment step based on invadopodia function, subcellular structures involved in cancer invasion into collagenous matrices [14–16], would serve to separate metastatic cells from all other cell types found in blood. Here we applied this functional cell separation method using a collagen adhesion matrix (CAM), modified as previously described [17,18], to enrich viable tumor cells from the blood of prostate cancer patients. In this study, we performed cellular analysis using fluorescence microscopy to identify CTCs that exhibit high avidity for and invasiveness into the extracellular matrix. Circulating cells that ingest fluorescently labeled CAM fragments (CAM+) are verified to be tumor cells by immuno-staining with common cytokeratin (CK) epithelial markers (Epi+) [5]. These epithelial markers are low or lacking in circulating leukocytes. CAM-enriched cells are viable and devoid of normal leukocytes [19–21] or apoptotic and necrotic cells [6,22,23] that may react with antibody staining non-specifically. In the present study, we hypothesize that certain CTCs exhibiting high avidity for, and invasiveness into the extracellular matrix, represent cancer cells disseminated into blood of prostate cancer patients. And, these cells are better indicators for metastasis and hormone responsiveness than the cells isolated using antibodies against epithelial surface antigens alone.

In addition, we examined the genomic profiles of CTCs enriched by CAM from blood of patients with CRPC by using oligonucleotide (oligo) array comparative genomic hybridization (aCGH). aCGH is a valuable tool for identifying clinically significant DNA copy number changes in tumor genomes, particularly in prostate cancer, which can then be examined for associations with clinical parameters [24–26]. Genomic gains and losses often coincide with genes crucial for tumor progression, and genomic loci with aberrant copy number can lead to better clinical diagnostics and prognostication [27,28]. Recently, oligo aCGH was shown to yield high resolution gene copy number mapping information of solid tumors [29,30]. This facilitates the identification of candidate genes mapping to a locus of altered dosage. Using aCGH, we previously found a significant concordance between the copy number changes in primary prostate tumors and unmatched metastatic tumors [31]. Also, copy number changes of matched primary prostate tumor and hormone naïve lymph node metastasis are almost identical, suggesting that this method could be used for detecting genomic biomarkers with associated metastatic phenotypes [32]. Based on these observations, we hypothesize that a subset of copy number aberrations representative of a primary tumor and its metastatic lesions can be identified in CTCs, and thereby potentially extending the clinical utility of such a biomarker.

2. Materials and methods

2.1. Patients and healthy blood donors

Twenty-one patients with metastatic CRPC, 13 patients with castration sensitive localized or metastatic prostate cancer (CSPC) prior to androgen deprivation, and 20 healthy male subjects were recruited for blood donation at the Stony Brook University Medical Center and the Veteran Administration Medical Center at Northport, NY. Similarly, another 13 patients with progressive metastatic CRPC and two healthy male individuals at the UCSF Urologic Oncology Clinic underwent blood sampling were recruited after written, informed IRB approved consent was obtained.

2.2. Blood sample preparation

Between 14 and 20 ml (median = 16 ml) of peripheral blood were collected in Vacutainer™ tubes (Becton Dickinson, Franklin Lakes, NJ; green top) with lithium heparin as the anticoagulant. Blood samples were delivered to the laboratory at room temperature within one to four hours from collection, and processed immediately.

Aliquots of 3 ml of whole blood were subjected to one of the two CAM-initiated rare cell enrichment methods. *CAM fractionation:* Blood samples were transferred into a CAM-coated tube (Vita-Cap™, Vitatex Inc., Stony Brook, NY) and incubated for 3 h at 37 °C while rotating at 10 rpm to imitate blood flow and maximize the contact between cells and the CAM substrate. The tubes were washed with 37 °C phosphate buffered saline (PBS) media to remove non-adherent cells. Tumor cells were eluted from the tube using collagenase and either used directly for microscopy, or DNA of CAM-bound cells was extracted for oligo aCGH. *Mono-nucleated cell (MNC) – CAM fractionation:* Blood samples were subjected to Ficoll density gradient centrifugation to obtain the MNC. MNC were then seeded onto a 16-well chamber slide coated with CAM containing fluorescently labeled type I collagen (Vita-Assay™, Vitatex Inc., Stony Brook, NY) for 12 h and the non-adherent cells were washed away with PBS to generate the remaining red fluorescent CAM-labeled cells.

2.3. Cell spiking experiments

To determine the effectiveness of CAM rare cell enrichment methods, model experiments were conducted in which 3–3000 PC3 tumor cells (as pre-determined by flow cytometry), labeled with PKH67 green fluorescence (Sigma, St. Louis, MO) were spiked into 3 ml samples of blood from healthy donors. The human prostate cancer cell line, PC3, was purchased from American Type Culture Collection (ATCC, Rockville, MD). The spiked fluorescent cells were recovered by both CAM and MNC-CAM methods and evaluated with fluorescence microscopy. In parallel, tumor cells were recovered by immunomagnetic cell separation using the Dynal Cellection™ Epithelial Enrich system (Invitrogen, Carlsbad, CA) for additional comparison.

2.4. Tumor cell enrichment and CAM ingestion labeling

To determine the invasive phenotype of CTCs, MNC from 0.5 ml whole blood aliquots were seeded onto one well of a red fluorescent CAM-coated 16-well chamber slide (Vita-Assay™, Vitatex Inc., Stony Brook, NY). Cells were incubated for 12–18 h to label the captured cells *in vitro*. This step labels tumor cells by making use of their ability

to ingest red fluorescent CAM fragments. Cells were then fixed with 3.5% paraformaldehyde/PBS, permeabilized with 0.1% Triton X-100, and subsequently immuno-stained using a mixture of green fluorescent antibodies against pan-CK (cytokeratins 1, 4, 5, 6, 8, 10, 13, 18, 19: clones C11 and others, Sigma, St. Louis, MO), epithelial cell adhesion molecule (EpCAM, clone Ber-Ep4, DakoCytomation, Carpinteria, CA), and epithelial surface antigen (ESA, clone B29.1, Biomeda, Foster City, CA) for analysis by digital fluorescence microscopy imaging. In addition, CAM-enriched cells were stained with an anti-CD45 antibody (clone T29/33, DakoCytomation, Carpinteria, CA) and followed by red color alkaline-phosphatase–anti-alkaline-phosphatase (APAAP) conjugated secondary antibodies (DakoCytomation, Carpinteria, CA) for internal control of cells with common leukocyte markers and normalization of fluorescence signal. Thus, CTCs were identified as circulating cells that ingested CAM and were stained with anti-epithelial antibodies (CAM+Epi+). In a CRPC patient, 89% of CAM+Epi+ cells were also positive for prostate specific antigen (PSA), suggesting a possibility to identify prostate CTCs as CAM+PSA+ cells. A Nikon E-400 inverted fluorescence microscope equipped with a Microfire digital camera system and Image Pro Plus software were used to examine and analyse the image results.

2.5. Cell culture

Blood samples were subjected to Ficoll density gradient centrifugation to obtain the mono-nucleated cells (MNC). The MNC from 0.5 ml whole blood aliquots were seeded onto one well of CAM-coated 96-well microtiter plate (Vita-Assay™, Vitatex Inc., Stony Brook, NY) for 12 h and the non-adherent cells were washed away by removing media and replacing with fresh media. Cells were cultured with CCC media (1:1 mixture of Dulbecco's modified Eagle's medium and RPMI1640 medium supplemented with 10% calf serum, 10% Nu-serum, 2 mM L-glutamine, 1 unit/ml penicillin, and 10 μg/ml streptomycin). In five of eight cases of patients with metastatic CRPC, tumor cells from blood were successfully cultured for 10 days and developed approximately 80 cellular colonies of epithelioid morphology per 1 ml of blood. Judging from approximately 72 cells per colony, the average proliferation rate of CTCs captured by CAM is 40 h.

2.6. DNA extraction and whole genome amplification

After performing the CAM tumor cell enrichment, DNA was extracted using the Wizard DNA Purification Kit (Promega, Madison, WI). The manufacturer's whole blood protocol was followed for the CAM− fraction (white blood cells, WBC) and the tissue culture cell protocol was followed for the CAM+ fraction (CTCs). The DNA quality was visualized by gel chromatography and quantified by a Nanodrop UV–Vis spectrophotometer (ThermoScientific, Wilmington, DE). 500 ng of high molecular weight DNA was used for oligo aCGH.

Whole genome amplification of DNA isolated from CAM-enriched cells with yields less than 500 ng was carried out using the GenomePlex Whole Genome Amplification Kit (Sigma, St. Louis, MO). 100 ng of genomic DNA was fragmented and then amplified according to the manufacturer's instructions. Amplified products were then column purified using the QIAquick Purification Kit (Qiagen, Valencia, CA), as per the manufacturer's protocol. Whole genome amplification was necessary for one of the nine patient's CTC DNA as the yield was less than 500 ng. Whole genome amplification, when necessary, does not introduce unacceptable copy number artifacts as determined using aCGH [33].

2.7. Oligonucleotide aCGH (oligo aCGH)

Oligo aCGH experiments were performed with Agilent's 244 K oligonucleotide arrays using 500 ng genomic DNA according to Agilent's protocols. Commercial male DNA obtained from blood of healthy donors (Promega, single lot), served as reference DNA for all oligo aCGH hybridizations. Agilent's Feature Extraction software was used to extract feature level data (e.g., signal intensities). All oligo aCGH data was analysed through the assistance of the UCSF Helen Diller Family Comprehensive Cancer Center's Biostatistics Core. We have previously optimized copy number assessment algorithms for high density oligonucleotide arrays and have found that a combination of circular binary segmentation (CBS) [34] and median absolute deviation (MAD) yield the best results (unpublished data). The array CGH data was segmented using CBS to translate experimental intensity measurements into regions of equal copy number. To enumerate genomic aberrations, the experimental variability (sample MAD) of each aCGH profile was estimated by taking the scaled MAD of the difference between the observed and segmented values. A clone was declared gained or lost if its absolute segmented value exceeded the sample MAD for a given profile. Copy number profiles for CTCs of prostate cancer patients were compared to each other and when appropriate to that of their matched primary, metastatic tumor(s) and/or white blood cells.

3. Results

3.1. Enrichment of rare prostate tumor cells from whole blood by CAM

The efficiency of isolating prostate tumor cells using CAM was determined by spiking a specified number of fluorescently labeled tumor cells into whole blood and quantifying their recovery rate using fluorescence microscopy (Fig. 1). Approximately 1–1000 PC3 prostate cancer cells (estimated using flow cytometry) were spiked into 1 ml of whole blood derived from a healthy donor and subjected to either a one-step CAM enrichment (Fig. 1A, CAM) or a two-step MNC-CAM enrichment in which whole blood was pre-processed using Ficoll density gradient centrifugation (Fig. 1A, MNC-CAM). PC3 cells in blood are CAM avid and five times larger than the circulating non-tumor cells such as white blood cells (Fig. 1A, double arrows indicating tumor cells and single arrows WBCs). Efficient recovery of fluorescently labeled PC3 cells was observed (Fig. 1B, CAM: 50, 26–73 [% mean, range]; MNC-CAM: 81, 58–100), even at the lowest concentration of ten spiked tumor cells. Recovery rates were higher in MNC-CAM than CAM enrichment methods, but were comparable under both conditions (Fig. 1B, $r^2 = 0.99$), suggesting a potential steric hindrance of red blood cells in the tumor cell-CAM contact. In comparison with anti-epithelial antibody purification, the efficiency of CAM recovery of tumor cells was nearly a hundred times higher than using Dynal Cellection™ Epithelial Enrich. Only a 0.27% ($N = 12$) recovery rate was observed when 1000 PC3 cells were spiked and recovered using an anti-epithelial antibody method (Fig. 1B). Surpris-

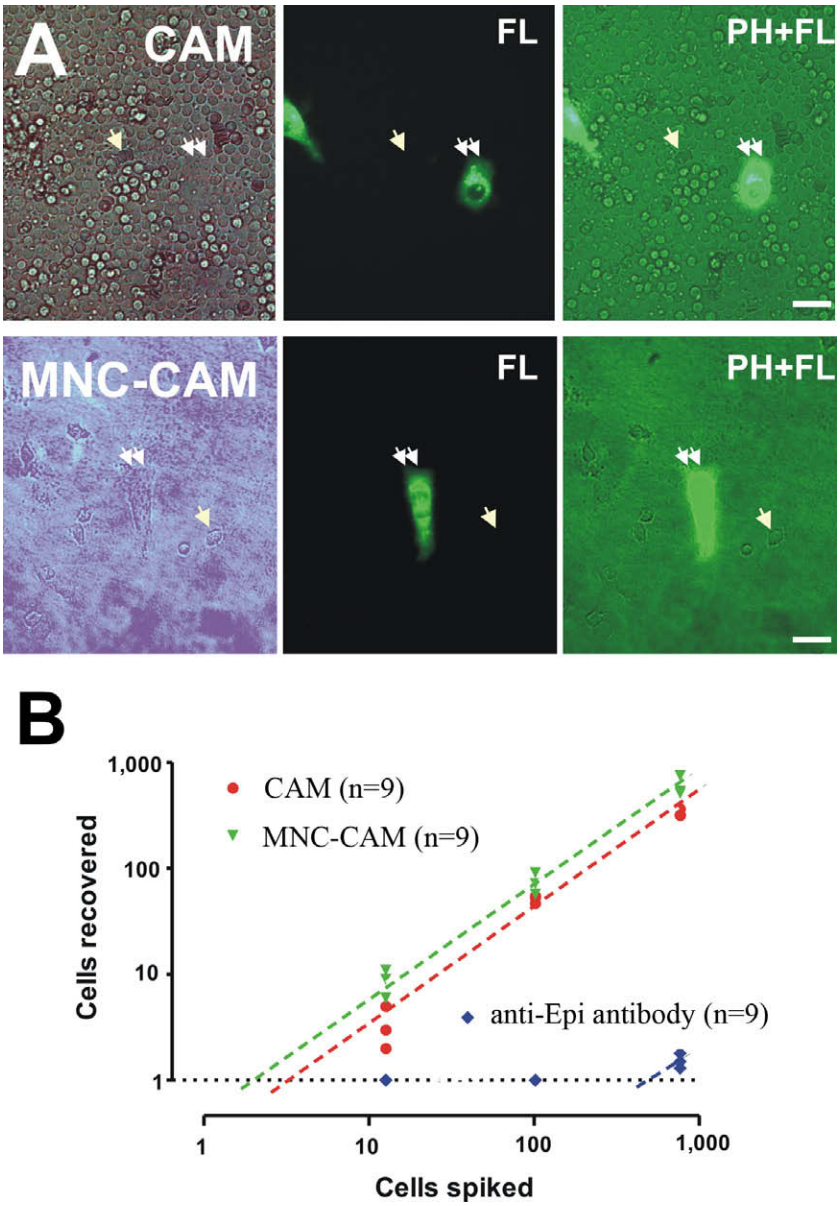

Fig. 1. Enrichment of prostate tumor cells from blood by CAM cell separation methods: (A) approximately 1–1000 fluorescently labeled PC3 cells were spiked into 1 ml of whole blood derived from a healthy donor, and recovered by either one-step CAM enrichment or two-step MNC-CAM enrichment assays. Double arrows indicate recovered tumor cells. Single arrows show co-isolated white blood cells. PH denotes phase contrast microscopy and FL is fluorescence microscopy. Photographs labeled PH + FL indicate the super-imposed image of the same field. Bar = 10 μm and (B) comparison of the yields of PC3 carcinoma cells isolated from blood using the CAM or MNC-CAM protocols and using the Dynal CELLection™ Epithelial Enrich Kit. Fluorescently labeled PC3 cells were spiked into blood samples from healthy donors, followed by one of the cell separation protocols. Cell enumeration was done with FL microscopy.

ingly, when cells derived from different tumor cell lines were used to perform epithelial cell capture by others using other devices and methods, [6,11] 100% of the spiked tumor cells were recovered. This apparent discrepancy between our antibody-captured cell result and previous antibody-based methods [6,11] could be explained by: (a) a possible down regulation of targeted epithelial surface antigen in the PC3 cells used in our study; (b) sophisticated devices and procedures used in previous antibody-based methods. Nevertheless, the CAM-initiated tumor cell enrichment performs well for whole blood and pre-processed MNC fraction, and both CAM methods achieve a high recovery rate similar to the recent CTC-chip epithelial antibody method [11].

3.2. Viability and proliferative propensity of cancer cells enriched by CAM

To evaluate the metastatic propensity of the cell capture from CRPC patients, we conducted a series of experiments in which MNC-CAM fractions were tested for cell viability, proliferative potential and immunoreactivity with anti-CK antibodies (Fig. 2) [35]. We verified the viability of tumor and "normal" cells from blood of CRPC patients, pre- and post-CAM enrichment, by using Molecular Probes LIVE/DEAD Viability Kit #4 (Invitrogen, Carlsbad, CA: live cells stain green fluorescence with calcein AM and dead cells stain red fluorescence with ethidium homodimer-1). Prior to CAM enrichment, less than 9% of cells in blood samples were via-

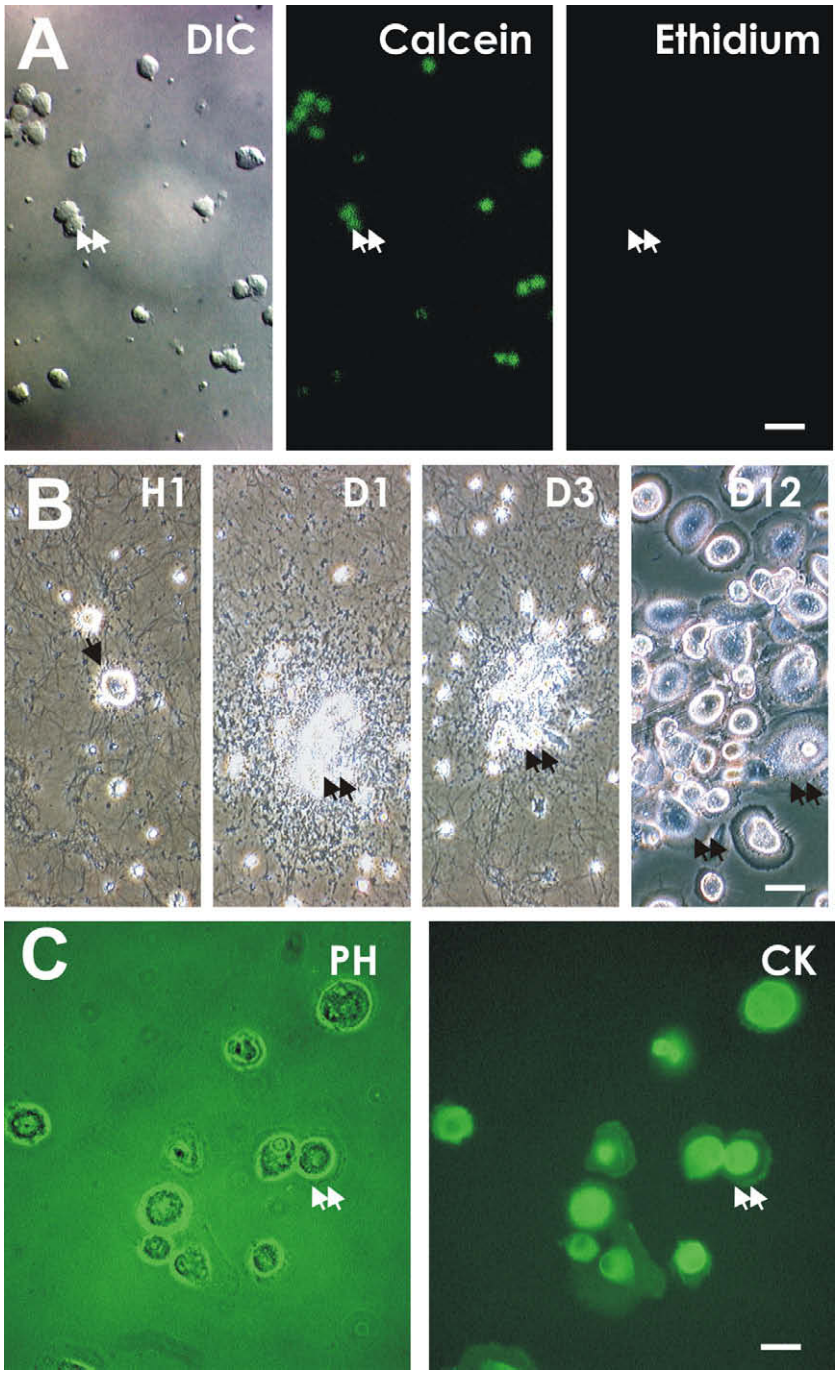

Fig. 2. Viability, proliferative properties and CK immuno-reactivity of CAM-enriched cells from CRPC patients: (A) live green fluorescent cells and lack of dead red fluorescent cells in fractions post-CAM enrichment. Bar = 60 μm; (B) CAM-enriched cells isolated from CRPC patients were cultured on the fibrous CAM scaffold for one hour (H1), one day (D1), three days (D3) and 12 days (D12). Live cells were photographed under phase contrast (PH) microscopy. Tumor cells grew as time increased; from H1 to D3, cells were seen as solitary (arrows) and afterward became clustered epithelioid cells (double arrows). Bar = 60 μm and (C) cells cultured for 12 days shown under PH microscopy (double arrow) and fluorescence microscopy for staining with antibodies against pan-cytokeratins (CK, double arrow). Bar = 30 μm.

ble (results not shown). However, post-CAM enrichment, greater than 99% of cells was viable (Fig. 2A). Overall, cell viability increased greater than 10-fold after CAM enrichment.

In addition, CAM-captured cells were readily cultured on the fibrous CAM scaffold *in vitro* (Fig. 2B). Tumor cells isolated by CAM from blood of CRPC patients were solitary and became epithelioid, producing colonies of CK+ epithelial cells from 1 week to 3 months (Fig. 2C). In contrast to CTCs captured by anti-epithelial antibody systems observed previously, [6,11] we have noticed no morphological feature that distinguishes CTCs from other circulating cells enriched by CAM, i.e., CTCs were seen to be heterogeneous in size and shape (Figs. 2A and 3A). However, CTCs cultured for more than 7 days exhibited epithelioid morphology (Fig. 2B

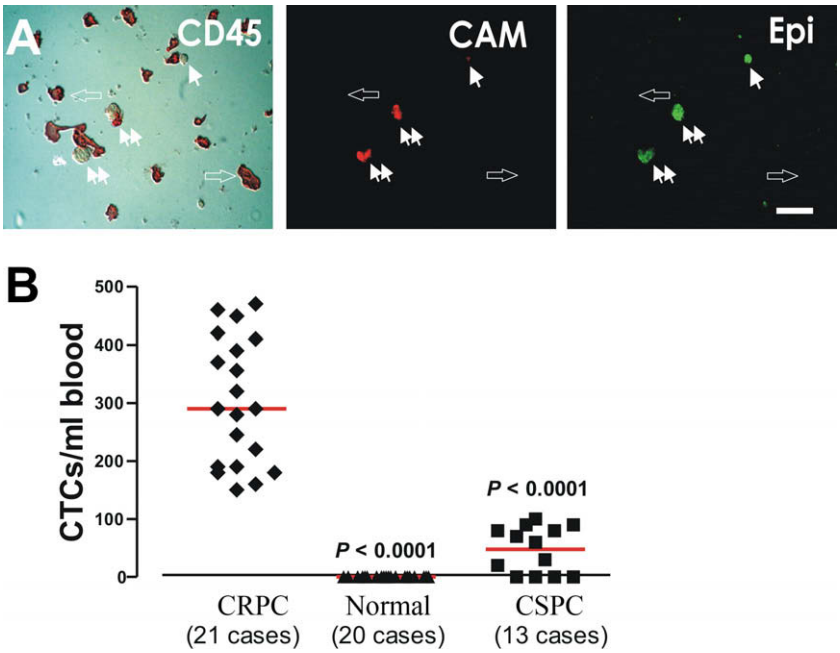

Fig. 3. CTC characterization based on the expression of CK epithelial lineage antigens and cellular uptake of CAM: (A) detection of CD45−CAM+Epi+cells (double arrows) as CTCs in a patient with metastatic CRPC. Circulating epithelial cells are CD45−CAM−Epi+ (single arrows), and leukocytes are CD45+CAM− (open arrows). Bar = 20 μm and (B) correlation between the number of CTCs in one ml of blood for 21 patients with metastatic CRPC, 13 CSPC and 20 healthy donors. Cells were counted manually under differential contrast interference/epifluorescence microscopy. The bars represent the median values in each subgroup of blood samples.

and C) that was distinct from the remaining hematopoietic cells. We thus concluded that CAM enriches cells from blood of CRPC patients that are viable and exhibit tumor progenitor propensity.

3.3. Characterization of CTCs isolated by CAM from CRPC patients

Having optimized the MNC-CAM enrichment with controlled quantities of prostate tumor cells, we tested its capacity to capture CTCs from whole blood samples donated by prostate cancer patients. A total of 34 samples from 34 patients with prostate cancer including metastatic CRPC ($n = 21$) and CSPC ($n = 13$) were studied. The average volume of blood analysed was 3.5 ml per sample (range, 2.2–6.5 ml), and aliquots of 0.5 ml per well of a 16-well chamber slide were stained with a specific antibody mixture. We also examined samples from 20 healthy male individuals (3.0 ± 0.5 ml [mean ± s.d.] of blood per subject) as controls.

CTCs captured from blood samples of cancer patients were identified using the invasive phenotype of cancerous cells, i.e., labeling tumor cells by their ability to ingest red fluorescent CAM fragments (see Methods and methods for detailed procedures); cells were identified by uptake of fluorescent CAM and co-staining with green fluorescein-conjugated anti-CK antibodies for epithelial cells (Fig. 3A, double arrows) to distinguish them from the circulating epithelial-like cells (Fig. 3A, single arrows) that were often observed in blood samples of healthy individuals. Cells labeled by ingested CAM and stained with anti-CK antibodies (CAM+Epi+) were scored as CTCs, whereas CD45+ cells were scored as contaminating normal circulating cells.

CTCs were identified in 21 of 21 (100%) patients with metastatic CRPC and in nine of 13 patients with CSPC. The number of CTCs isolated ranged from 150 to 740 per ml for CRPC (322 ± 143 [mean ± s.d.] CTCs per ml) and 0–100 (48 ± 40) for CSPC patients (Fig. 3B). None of the 20 healthy subjects had any identifiable CTCs; two BPH patients had no CTCs. We calculated the sensitivity (100% for CRPC and 69% for CSPC) and specificity (100%) of the CAM-initiated CTC detection for prostate cancer. We also evaluated the reproducibility of CTC capture using split samples and showed high experimental reproducibility ($r^2 = 0.98$, $n = 5$). Thus, the CAM cell separation platform provides a highly enriched population of CTCs enabling the identification of CTCs in subjects with metastatic and clinically localized prostate cancers and warrants further clinical study.

3.4. Genetic analysis of CRPC CTCs isolated by CAM

To determine whether CAM-captured cells are suitable for subsequent genetic analyses, we performed genomic copy number profiling of blood samples from thirteen patients with CRPC. The DNA extraction procedure was performed on the CAM fraction (CTC sample) and the MNC fraction from cells left unbound by CAM (WBCs). DNA was isolated from the CAM fraction for 12 of the 13 patients. The yield of one of the twelve samples (CTC3) was too low to perform oligo aCGH, so it was amplified (aCTC3). All DNAs were evaluated by gel chromatography (Fig. 4A), except CTC1 which only had enough DNA for oligo aCGH. Three of the CTC DNAs were very degraded (CTC3, 5, 10) as evident by the presence of a DNA smear and the lack of a high molecular weight band (Fig. 4A).

Oligo aCGH was performed for all CTC DNAs and a subset ($N = 3$) of matching WBC DNAs. It should be noted that all of the nine CTC samples with high molecular weight DNA performed well on the microarrays, by exhibiting good fluorescence signal to noise ratios. The three degraded CTC samples (CTC3, 5, 10) were subjected to an oligo aCGH protocol for degraded samples (Agilent part #5190–0419), but still did not pass Agilent's quality control metrics. To evaluate the reproducibility of the Agilent data, the average standard deviation of the $\log_2$ratios for 1000 randomly dispersed replicate probes on an array was calculated. For the nine CTCs with acceptable oligo aCGH, the average standard deviation was 0.02.

Matched CTC and WBC DNAs for three cases were profiled by oligo aCGH and compared. The percentage of the genome that was aberrant was greater in the CTC fraction versus the MNC fraction for the three matched cases studied, displaying the difference without making any assumptions of the underlying statistical distribution (Fig. 4B). One of the CRPC cases (CTC13) had primary and metastatic tissue and a second case (CTC8) had primary tumor material available for research purposes. Copy number profiles of these matched CTC-primary/metastatic cases were compared and found to be similar (Table 1). A Kappa score approaching the value one suggests a high degree of similarity. Oligo aCGH data for all tumor samples are available in Supplementary data.

The summary of copy number changes for each locus across the nine CTC samples is displayed in Fig. 5. A detailed list of these alterations and the probes are supplied in Supplementary data. Noteworthy, recurrent

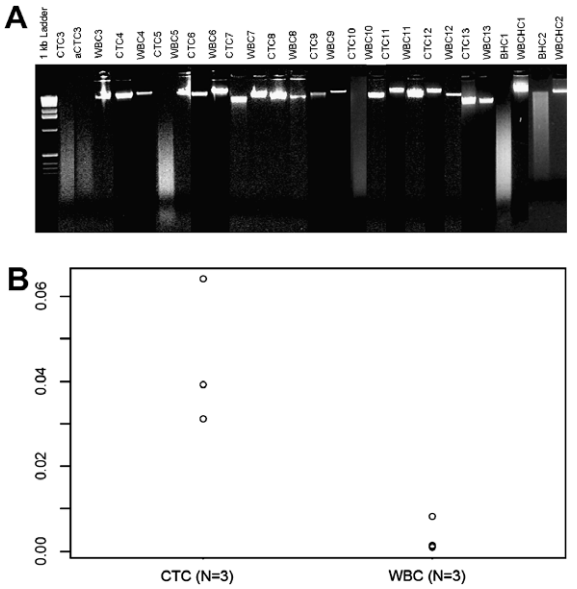

Fig. 4. (A) DNA quality visualized by gel chromatography. 100–200 ng of DNA from cells isolated with the Vitatex one-step VitaCap tubes was loaded into each gel lane. The CAM+ fraction from 11 patients is paired with the matching CAM− fraction (WBC). aCTC represents the amplified sample. Two healthy controls were included, with the CAM+ fractions denoted as BHC1 and BHC2, and their matching CAM− fractions as WBCHC1 and WBCHC2. A 1 kb ladder was run for molecular sizing. The smears for the CAM+ fraction in the healthy controls, suggests that patients three, five and ten may not have had CTCs and (B) comparison of copy number changes in matched CTCs and WBCs. The fraction of the genome that is altered is plotted on the *y*-axis.

Table 1
Metric comparing copy number changes in matched primary, metastatic and circulating tumor cells.

Sample comparison	Kappa score
Met13 versus CTC13	0.84
CTC13 versus RP13	0.90
MET13 versus RP13	0.91
CTC8 versus RP8	0.81

changes were detected. Some cancer related genes mapped to the loci aberrant in 50% or more of the CTC samples. These included POTE15 (protein expressed in prostate, ovary, testis, and placenta 15), similar to ADAM5 (disintegrin and metalloprotease domain 5), cyritestin protein, SUSD4 (sushi domain containing four) and GSTT1 (glutathione S-transferase theta 1). Considering the high number of captured viable CTCs, the CAM CTC enrichment method provides a powerful opportunity for CTC-based genomic analyses.

4. Discussion

We have applied a CAM based functional cell separation method to obtain sufficient quantity and quality of viable CTCs for cellular analyses and genomic profiling of prostate cancer. The CAM method of tumor cell enrichment is distinctive among current CTC enrichment technologies [4,5] in that it readily isolates viable tumor cells on a platform enabling further cellular and molecular analyses as shown in this report. Cell culture plates or tubes coated with collagen-based CAM scaffolds allow blood cells to be washed out, resulting in a 10^6-fold enrichment, which is the highest cell enrichment among existing technologies. Viable CTCs concentrated on CAM scaffolds are readily extracted with DNA and RNA buffers for molecular analyses of cancer metastasis or treated with collagenases to make into cell suspension for cellular assays such as flow cytometry and fluorescence microscopy.

Currently, no consensus has been reached on which methods should be best to detect CTCs in epithelial cancer patients. Traditionally, it was believed that epithelial cancers spread primarily through the circulation and the presence of epithelial cells in blood or bone marrow could be an indicator of metastatic cancer cells. Cells in blood captured by antibodies against epithelial surface antigens augmented with sophisticated devices have been a popular means to detect CTCs [4–7]. However, some research has shown that epithelial cells may be present in blood of healthy populations, either as non-specific epithelial antigen binding to leukocytes, or as benign epithelial cells in circulation [4,5]. Additionally, recent work investigating the role of epithelial mesenchymal transition in cancer metastasis has found a heterogeneous downregulation of epithelial surface antigens in invasive epithelial tumor cells [5,19–21], thus reliance on a few standard epithelial markers alone for identification of tumor cells may not be reliable. Furthermore, current detection techniques do not distinguish viable cells from non-viable cells. The lifespan of a CTC is limited by anoikis [3–7,22,23], thus a proportion of epithelial-recovered CTCs may represent dead or dying cells which may not contribute greatly to cancer spread.

To address these concerns, we evaluated the feasibility of using a CAM-initiated CTC assay that enriches cells based on their invasive properties in addition to their expression of epithelial cell markers. We reason that detection of these *invasive* CTCs may offer an alternative method to assess metastatic progression that may be of prognostic value. In this exploratory study, we found that CRPC patients have 322 ± 143 [mean ± s.d.] CTCs per ml of blood, a number that is three times higher than was reported using a sensitive anti-epithelial antibody CTC-chip technology [11]. We also found that there are 48 ± 40 [mean ± s.d.] CTCs per ml of blood in CSPC patients. Whether this increase in CTC detection is a result of increased production or release of tumor cells into blood, or decreased CTC death or clearance remains to be determined. Regardless, these findings suggest a clinical utility for quantifying invasive CTCs in monitoring cancer progression.

Copy number profiling DNA from blood yields good fluorescence signal to noise ratios and thereby improves the confidence of determining which loci are altered. This is important when sufficient DNA is not available for replicate experiments and monetary constraints. Also, DNA copy number changes in the blood are less variable day to day, as opposed to RNA. The DNA from the bound fraction for the healthy controls was always degraded (Fig. 4A; BHC1, BHC2). The DNAs from the CAM fractions were not always high molecular weight gel bands (Fig. 3; CTC3, 5, 10) suggesting that these specimens did not con-

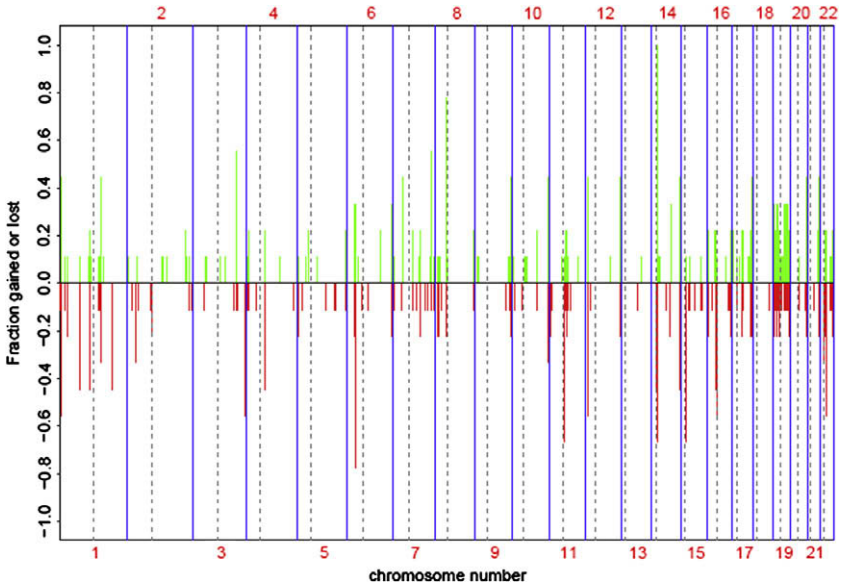

Fig. 5. CRPC CTC copy number changes. Copy number aberrations for the nine CTCs with high mw DNA (see Fig. 4A) and that passed Agilent's quality control metrics is shown versus the chromosomal position. Gains are shown in green and deletions in red. Recurrent changes between patients were observed and may represent loci associated with CRPC.

tain sufficiently high number of CTCs. Therefore, we focused on the remaining CTC samples for analysis.

Although a common concern with assessing copy number profiles of CTCs is that WBCs may contaminate the sample (i.e., the CAM fraction in our approach), this should not interfere significantly here. First, aCGH of tumor samples has been shown to allow up to 60% contaminating normal DNA while still providing accurate copy number information for the tumor [36]. Secondly, our cellular analysis shows that CAM-enriched cells are greater than 99% viable, proliferate into CK+ cell colonies, and exhibit ability to degrade and ingest collagenous matrices, characteristics of the tumor invasive phenotype [15,16]. Although WBCs possess germline changes, the CTCs should contain additional tumor acquired alterations. The fraction of the genome that was altered was always greater for the CTC fraction when compared to matched WBC sets (Fig. 4B), providing further evidence that we are indeed isolating tumor cells from the blood. Recurrent changes in the matched CTC and MNC cell fractions could be related to either varying levels of WBC contamination in the CAM fraction and/or to germline changes which could be informative. Recently, germline copy number variants (CNVs) have been identified, and may contribute to a significant proportion of normal genetic variation in humans [37,38]. Since these CNVs often overlap genes, they may impact gene expression and cause or confer risk to complex diseases such as cancer. Changes seen in only one fraction are believed to be either unique to the CTC or the matching WBCs.

Our prior work suggests that primary prostate and metastatic lesions possess similar copy number changes [31,32]. In line with this observation, the matched primary and metastatic tumors for CTC13 show such concordance (Table 1). This observation was extended to the CTCs, which showed similar copy number changes to their matched primary and/or metastatic tumor DNAs (Table 1). This provides additional evidence that the CTCs are indeed being isolated with this CAM based approach.

Cancer related genes were identified when focusing on genes altered in 50% or more of the CTCs. Loss of the POTE15 gene was frequently detected in the CTCs. Consistently, differential expression of POTE related genes has been observed in prostate cancer [39]. The GSTT1 locus was frequently deleted in the CTCs. GST enzymes function in the detoxification of electrophilic compounds, including carcinogens, therapeutic drugs, environmental toxins and products of oxidative stress. Therefore, loss of GSTT1 could make a cell more susceptible to malignant transformation, and less likely to respond to therapeutic intervention. GSTT1 expression has been reported to be significantly lower in CRPC tumors as compared to untreated primary tumors [40]. Loss of the SUSD4 gene was frequent in the CTC samples. Although the function of SUSD4 is not known, the expression of SUSD4 was significantly decreased in metastatic tumors compared to primary prostate tumors and normal prostate tissue [41]. An ADAM5-like protein and cyritestin (part of the ADAM family of proteins) were frequently gained in the CTCs. ADAM enzymes cleave extracellular portions of transmembrane proteins. In breast cancer, ADAM10 was found to activate the HER2 receptor, thus promoting tumor growth [42]. The sample size is too small to draw conclusions about the genes altered, although these preliminary findings are provocative.

Tumor cells were demonstrated to be recovered from the circulation by CAM, which facilitated subsequent genomic analyses. This approach allows one to go beyond enumeration of CTCs and warrants further studies aimed at CTC genomic profiling for identification of potential bio-

markers. To the best of our knowledge this is the first time CTCs have been profiled by oligo aCGH. Since blood samples are easy to obtain, this report shows that CTCs may represent a largely untapped resource for studying metastatic prostate cancer. As castration resistance ultimately leads to death in metastatic CRPC, new tools such as this will give insight into hormone resistance in prostate cancer and will delineate important pathways that may be targeted therapeutically.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2008.12.007.

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